Developing assisted reproductive technologies for endangered North American amphibians

By

Cecilia Jane Langhorne

A Dissertation
Submitted to the Faculty of Mississippi State University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Animal Physiology in the Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology

Mississippi State, Mississippi

May 2016
Developing assisted reproductive technologies for endangered North American amphibians

By

Cecilia Jane Langhorne

Approved:

______________________________
Scott T Willard
(Major Professor/Graduate Coordinator)

______________________________
Andrew J Kouba
(Committee Member)

______________________________
Peter L Ryan
(Committee Member)

______________________________
Jean M Feugang
(Committee Member)

______________________________
William V Holt
(Committee Member)

______________________________
George M Hopper
Dean
College of Agricultural and Life Sciences
An alarming number of anuran (frog and toad) species are facing the threat of extinction in the wild. In efforts to address this conservation crisis, captive breeding programs are rapidly being established at zoos and research institutions worldwide. However, the captive management of anurans can be challenging, as their reproduction is a tightly regulated hormonal response to environmental stimuli, often unknown or absent in captivity. Consequently, ex-situ breeding efforts tend to be greatly hindered by a paucity of knowledge in anuran reproductive physiology and, for many species on the brink of extinction, time is running out. Assisted reproductive technologies (ARTs), such as exogenous hormone induction of gamete release, artificial fertilization for population augmentation, and cryopreservation for the long-term storage of genetics, have the potential to greatly enhance captive breeding efforts in lieu of natural breeding.

Broadly, research aims were to develop assisted reproductive technologies for captive populations of the declining Southern Rocky Mountain boreal toad (Anaxyrus boreas boreas), the critically endangered Mississippi Gopher Frog (Lithobates captio sevosa) and Puerto Rican crested toad (Peltophryne lemur). Specific objectives were to a)
trial the efficacy of exogenous hormone treatments on sperm release in male target species by characterizing spermiation response across time; b) investigate methods for increasing sperm longevity through cold-storage and cryopreservation techniques; c) ascertain motility recovery rates and functional capacity of cold-stored and frozen-thawed spermatozoa through artificial fertilization techniques, and; d) apply successfully developed ARTs to determine the feasibility of genetically linking *in-situ* and *ex-situ* populations of *A. b. boreas*, through artificial fertilization of male and female gametes from wild and captive toads, respectively. Research outcomes from this study include the successful development of exogenous hormone protocols, spermiation profiles and sperm cryopreservation techniques for all target species. Additionally, these studies enabled validation of an alternative method for increasing genetic diversity in captive anurans through *in-situ-ex-situ* gamete linkage. Overall, this research emphasizes the potential value of assisted reproductive technologies as conservation tools for supporting the recovery of endangered frog and toad species worldwide.
DEDICATION

This manuscript is dedicated to my family and friends for their unwavering support and encouragement in my quest to Save the Frogs, and to the following people: Prof. Roger Downie, who opened up the world of frogs to me all those years ago; Prof. David Houston who introduced me to the concept of cryopreservation for conservation; and Sarah Johnson and the NIMR crew for providing me with five wonderful years of ART training. Finally, but most significantly ($P<0.05$), I dedicate this research to the frogs, for their inspiring tenacity and for making our world a better place.

‘….the natural world is the greatest source of excitement; the greatest source of visual beauty; the greatest source of intellectual interest. It is the greatest source of so much in life that makes life worth living’ – Sir David Attenborough
ACKNOWLEDGEMENTS

I am expressly grateful to Scott Willard and Andy Kouba for providing me the opportunity to pursue my passion through this PhD, and for the many other opportunities that accompanied it. I would like to extend my sincerest gratitude to my whole committee: Scott Willard, Andy Kouba, Peter Ryan, Jean Feugang and Bill Holt, for guidance and support over the last five years (a special thanks to Bill for negotiating time-zones to serve on my committee). I would also like to thank all the staff at the Colorado Parks and Wildlife’s Native Aquatic Species Restoration Facility in Alamosa, CO, especially Ted Smith, Harry Crockett and Tom Mix, for all their help and hospitality over three wonderful Rocky Mountain summers. I am also grateful to the following CPW staff for their assistance with coordinating our fieldwork (and putting up the tent): Kevin Thompson, Scott Schneider, and Bradley Lambert. Many thanks also to the staff and keepers at the Memphis Zoo, Fort Worth Zoo, and Omaha’s Henry Doorly Zoo for their generosity and assistance. Finally, I would like to acknowledge my wonderful colleagues Natalie Calatayud and Katie Graham for not only being such outstanding lab mates but also delightful friends, Candace Williams (local tour guide/chauffeur/real estate agent) for helping me settle in, GB for getting me through that final stretch, and to the many other people who passed through during my time here, especially our wonderful student workers who so attentively cared for our colony of frogs and toads over the years:
Michael Robinson, Lindsay Bullock, Christy Steadman, Johnny May, Amanda Mullen, Andrew House and Mathew Christiansen and Allison Julien.
# TABLE OF CONTENTS

**DEDICATION** ........................................................................................................................ ii

**ACKNOWLEDGEMENTS** ........................................................................................................ ii

**LIST OF TABLES** ................................................................................................................ x

**LIST OF FIGURES** .............................................................................................................. xi

**CHAPTER**

I. **INTRODUCTION: AMPHIBIANS IN PERIL** .............................................................. 1

II. **LITERATURE REVIEW** ................................................................................................. 6

Frogs: Evolution’s great success story ............................................................ 6
  Life history and ecology .............................................................................. 7
  Hibernation and Estivation ...................................................................... 8
  Reproductive strategies ........................................................................... 10
Amphibian reproductive system ................................................................. 14
  The Hypothalamic-Gonadal-Pituitary (HPG) axis .................................. 14
    The gonadotropin-releasing hormone systems .................................. 17
    Modulation of the GnRH system by dopamine .................................. 17
    Gonadotropin hormones .................................................................. 18
  Testes and spermatogenesis .................................................................. 20
  Ovaries and ovulation ........................................................................... 21
  Anuran fertilization ............................................................................... 22
The Amphibian Extinction Crisis ................................................................. 23
  Global forces of destruction .................................................................. 25
  The importance of frogs ........................................................................ 25
    The ecological role of frogs ................................................................ 25
    Nature’s pharmacy ............................................................................. 27
  Global response to amphibian declines ................................................. 28
*Ex-situ* Conservation ................................................................................. 31
  Captive breeding programs ................................................................. 31
  Captive breeding challenges ................................................................. 33
  Anuran captive breeding ..................................................................... 34
  Reproductive failure in anuran captive breeding ................................. 36
    Absence of reproductive cues ......................................................... 37
Nutritional deficiency ................................................................. 38
Stress-induced inhibition of reproduction ................................. 39
Developing ARTs for Captive Breeding .................................... 40
Novel hormone therapies .......................................................... 41
The application of gonadotropin-releasing hormone agonists .... 43
The application of exogenous chorionic gonadotropins .......... 47
Artificial fertilization ................................................................. 48
Gamete cryopreservation ........................................................... 50
Cryoprotectant solution ............................................................. 51
The causes of cryoinjury ............................................................. 53
Gamete cryopreservation in the anurans ................................. 54
Spermatozoa cryopreservation .................................................. 54
Oocyte cryopreservation ........................................................... 58
Genome Resource Banking ....................................................... 59
The Use of Model Species for Research ...................................... 62
Model species: Fowler’s toad (Anaxyrus fowleri) ...................... 64
Target species: Boreal toad (Anaxyrus boreas boreas) ............. 66
Target species: Puerto Rican crested toad (Peltophryne lemur) .. 68
Target species: Mississippi gopher frog (Lithobates sevosa) ...... 70
References ................................................................................. 73

III. HORMONALLY-INDUCED SPERM RELEASE AND SHORT TERM
COLD STORAGE OF SOUTHERN ROCKY MOUNTAIN
BOREAL TOAD (ANAXYRUS BOREAS BOREAS)
SPERMATOZOA ........................................................................... 92

Introduction .................................................................................. 92
Materials and Methods ............................................................... 99
  Animals and spermic urine collection ...................................... 99
  Study 1: Characterizing spermiation response to three exogenous
  hCG treatments ........................................................................ 100
  Study 2: Short-term cold storage of hormonally-induced spermic
  urine ...................................................................................... 101
Statistical analysis ...................................................................... 102

Results ......................................................................................... 103
  Study 1: Characterizing spermiation response to three exogenous
  hCG treatments ........................................................................ 103
  Male response to hormone treatment .................................... 104
  Sperm production in response to hormone treatment ............. 106
  Spermatozoa motility parameters .......................................... 108
  Study 2: Short-term cold storage of hormonally-induced spermic
  urine ...................................................................................... 112
Discussion .................................................................................. 115
  Characterizing spermiation response to three exogenous hCG dose
  treatments ............................................................................. 116
Study 2: Short-term cold storage of hormonally-induced spermic urine .................................................................................................124
Conclusion ...................................................................................................126
Acknowledgements ...........................................................................................128
References .........................................................................................................129

IV. SPERMATION RESPONSE TO EXOGENOUS HORMONE THERAPY IN HIBERNATED AND NON-HIBERNATED SOUTHERN ROCKY MOUNTAIN BOREAL TOADS (ANAXYRUS BOREAS BOREAS) .................................................................135

Introduction .......................................................................................................135
Materials and Methods ......................................................................................139
Animals ........................................................................................................140
Non-hibernated boreal toad colony (NASRF) .......................................140
Hibernated boreal toad colony (MSU) ..................................................140
Comparison of spermic urine production between hibernated and non-hibernated boreal toads ............................................................141
Statistical analysis .......................................................................................142
Materials and Methods ......................................................................................143
Effects of hibernation on spermiation response ..........................................143
Spermiation response .............................................................................144
Sperm concentration ..............................................................................145
Discussion ..........................................................................................................150
Effect of hibernation on spermiation response ............................................150
Conclusion .........................................................................................................159
Acknowledgements ...........................................................................................160
References .........................................................................................................161

V. SPERMATOZOA CRYOPRESERVATION OF THREE BUFONID SPECIES: THE FOWLER’S TOAD (ANAXYRUS FOWLERI), THE SOUTHERN ROCKY MOUNTAIN BOREAL TOAD (ANAXYRUS BOREAS BOREAS), AND THE PUERTO RICAN CRESTED TOAD (PELTOPHRYNE LEMUR) ..............................................................................................165

Introduction .......................................................................................................165
Materials and Methods ......................................................................................171
Animals ........................................................................................................171
Fowler’s toad (Mississippi State University) ........................................171
Puerto Rican crested toad (Omaha’s Henry Doorly Zoo) .....................171
Boreal toad (Native Aquatic Species Restoration Facility) ...................171
Tadpoles ................................................................................................172
Spermic urine induction, collection and analysis ..................................172
Study 1: CPA Cryotoxicity evaluation ........................................................173
Cryoprotectant solutions........................................................................173
VI. SPERMIATION INDUCTION, CRYOPRESERVATION AND POST-THAW FERTILITY IN THE CRITICALLY ENDANGERED MISSISSIPPI GOPHER FROG (LITHOBATES SEVOSA)
VII. LINKING IN SITU AND EX SITU POPULATIONS OF A DECLINING TOAD SPECIES (ANAXYRUS BOREAS BOREAS) .............................................................................................................. 246

Introduction ....................................................................................................... 246
Methods ............................................................................................................. 251
Animals ............................................................................................................ 251
  Wild-caught boreal toads ........................................................................ 251
Study 1: In-situ spermiation induction and collection .................................. 251
  Spermic urine evaluation ........................................................................ 252
  Effect of short-term cold storage and cryopreservation on sperm quality parameters .......................................................................................................................... 253
  Sperm short-term cold storage ................................................................ 254
  Sperm cryopreservation ............................................................................ 254
Study 2: Effect of short-term cold storage and cryopreservation on sperm quality parameters ................................................................. 253
Study 3: Fertilization capacity of chilled and frozen-thawed sperm ........... 255
  Ex-situ oocyte recovery artificial fertilization (AF) ................................... 255
  Tadpoles ................................................................................................... 256
Statistical analysis ........................................................................................... 257

Results ............................................................................................................... 257
  Study 1: In-situ spermiation induction and collection ............................. 257
  Study 2: Effect of short-term cold storage and cryopreservation on sperm quality parameters ................................................................. 262
  Sperm short-term cold storage ................................................................. 262
  Sperm cryopreservation ............................................................................ 265
  Study 3: Fertilization capacity of chilled and frozen-thawed sperm ....... 266
Discussion ......................................................................................................... 268
Acknowledgements ....................................................................................... 273
References ....................................................................................................... 275

VIII. CONCLUDING REMARKS ........................................................................ 279
### LIST OF TABLES

<table>
<thead>
<tr>
<th></th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Statistical results for two-way ANOVA procedure of main effects and their interactions on sperm quantity and quality parameters</td>
</tr>
<tr>
<td>2</td>
<td>The composition of four trehalose-based CPA solutions with different levels of penetrating cryoprotectant</td>
</tr>
<tr>
<td>3</td>
<td>Effect of exposure to a cryoprotectant (CPA) solution on <em>Anaxyrus fowleri</em> sperm motility (MOT) and viability (VIA) recovery</td>
</tr>
<tr>
<td>4</td>
<td>Effect of sperm exposure to freeze-thawing on the developmental outcomes of <em>Anaxyrus b. boreas</em> embryos and tadpoles reared at 20°C</td>
</tr>
<tr>
<td>5</td>
<td>Summary of protocols for sperm cryopreservation of <em>Bufo</em>, <em>Rana</em>, <em>Eleutherodactylus</em> and <em>Xenopus</em> species</td>
</tr>
<tr>
<td>6</td>
<td>A review of slow cooling and thawing rates of cryopreserved sperm from a number of different anuran species</td>
</tr>
<tr>
<td>7</td>
<td>Hormone treatments for induction of ovulation and egg deposition in <em>L. sevosa</em> females treated with one of four exogenous hormone regimens</td>
</tr>
<tr>
<td>8</td>
<td>Total number of <em>L. sevosa</em> eggs from each breeding pair and the percentage cleavage rate after artificial fertilization with either fresh or frozen-thawed spermatozoa</td>
</tr>
<tr>
<td>9</td>
<td>Mean weight, SVL and sperm quality parameters in spermic urine samples of wild-caught male boreal toads from each breeding season</td>
</tr>
<tr>
<td>10</td>
<td>Sperm motility parameters from wild-caught male <em>A. b. boreas</em> before (pre-freeze) and after (post-thaw) cryopreservation</td>
</tr>
<tr>
<td>11</td>
<td>Percentage of embryos at cleavage and tadpole stage, and the total number of tadpoles completing metamorphosis in <em>A. b. boreas</em> artificial fertilization trials using chilled and frozen-thawed sperm</td>
</tr>
</tbody>
</table>

x
# LIST OF FIGURES

1. Hypothalamic-Pituitary-Gonadal Axis ..........................................................16
2. Exogenous hormone manipulation of the HPG axis.................................46
3. The evolution of anuran sperm cryopreservation 1776-2013 ....................56
4. The spermiation response of male boreal toads over 24 h following exogenous hormone administration ..................................................105
5. Sperm concentration released by boreal toads over a 24 h period in response to administration of 3 IU g\(^{-1}\), 10 IU g\(^{-1}\) or 15 IU g\(^{-1}\) hCG .................................................................107
6. Motility parameters of spermatozoa released by boreal toads over a 24 h period in response to administration of 3 IU g\(^{-1}\), 10 IU g\(^{-1}\) or 15 IU g\(^{-1}\) hCG .....................................................................................109
7. FPM Quality of spermatozoa released by boreal toads over a 24 h period in response to administration of 3 IU g\(^{-1}\), 10 IU g\(^{-1}\) or 15 IU g\(^{-1}\) hCG ..........................................................................................111
8. The percentage of boreal toad spermic urine samples maintaining motility over 14 days in cold storage at 4° C .........................................112
9. The influence of time on the Total Motility and Forward Progressive Motility of boreal toad spermatozoa maintained in cold-storage for up to 14 days .......................................................................................113
10. Regression analysis of the relationship between time in cold storage at 4° C and Total Motility (TM) and Forward Progressive Motility (FPM) exhibited by spermatozoa from the boreal toad over 14 days ...........................................................................115
11. Spermiation response of hibernated and non-hibernated male boreal toads at each collection time-point following exogenous hormone administration of 10 IU g\(^{-1}\) hCG .................................................................145
12 Mean sperm concentration released by hibernated and non-hibernated boreal toads over a 24 h period in response to administration of 10 IU g^{-1} hCG ..................................................................................146

13 Motility parameters of spermatozoa released by hibernated and non-hibernated boreal toads over a 24 h period in response to administration of 10 IU g^{-1} hCG ........................................................................148

14 FPM-Q of spermatozoa released by hibernated and non-hibernated boreal toads over a 24 h period in response to administration of 10 IU g^{-1} hCG ..................................................................................149

15 *A. fowleri* spermatozoa stained with fluorescent dyes (SYBR)-14\® and propidium iodide (PI) to ascertain membrane integrity .......................175

16 Sperm cryopreservation equipment for slow-rate freezing ......................177

17 HOBO\® DataLogger temperature log of the cooling rate of a cryosuspension loaded into a 0.25mL freezing straw ........................................178

18 Effect of freeze-thawing on the motility parameters of sperm from *Anaxyrus fowleri* cryopreserved in four different cryoprotectant solutions .............................................................................................184

19 Effect of freeze-thawing on the motility parameters of sperm from *Anaxyrus b. boreas* and *Peltophryne lemur* cryopreserved in four different cryoprotectant solutions ........................................186

20 Effect of freeze-thawing on the quality of forward movement of *Anaxyrus. Fowleri, A. b. boreas, and Peltophryne lemur* sperm cryopreserved in four different cryoprotectant solutions ..........................188

21 Effect of freeze-thawing on *Anaxyrus b. boreas* sperm motility parameters ..............................................................................................191

22 Effect of freeze-thawing on the percentage of *A. b. boreas* tadpoles ..........192

23 Spermiation response of male *L. sevosa* over a 2.5 h period in response to exogenous hormone administration of 500 IU hCG + 15\µg GnRHa ...........................................................................................................227

24 Spermatozoa TM and FPM of male *L. sevosa* over a 2.5 h collection period in response to exogenous hormone administration ..................228

26 Effect of freeze-thawing on the motility parameters of spermatozoa from *L. sevosa* ......................................................................................229
27 Percentage motility of spermatozoa from fresh spermic urine samples (white bar), and percent TM (striped bar) and FPM (grey bar) recovered from frozen-thawed male _L. sevosa_ spermatozoa ..............231

28 The quality of forward motile _L. sevosa_ sperm before (white bar) and after (grey bar) cryopreservation in a DMFA-based cryoprotectant .................................................................232

29 Percentage of fertilized embryos to reach cleavage, tadpole and metamorph stage following artificial fertilization with fresh and frozen-thawed _L. sevosa_ sperm.................................................................235

30 Embryonic development of eggs fertilized using frozen-thawed _L. sevosa_ sperm ..................................................................................................................236

31 Collection, processing and cryopreservation of boreal toad spermatozoa under field conditions .................................................................253

32 Spermiation response (A) and sperm concentration (B) of wild-caught male _A. b. boreas_ over 5 h following exogenous hormone administration of hCG.................................................................260

33 Mean TM and FPM (A), and FPM-Q (B) of spermatozoa from wild-caught male _A. b. boreas_ over 5 h following exogenous hormone administration of hCG .................................................................261

34 Field locations of in-situ sperm collections during breeding seasons 2012, 2013 and 2014 ..........................................................................................262

35 TM (A), FPM (B) and FPM-Q (C) of sperm from wild-caught male _A. b. boreas_ after cold-storage at 4°C for up to 96 h post collection..................................................................................264
CHAPTER I
INTRODUCTION: AMPHIBIANS IN PERIL

Earth is facing its largest mass extinction in the history of humanity (Wake & Vrendenberg, 2008) with potentially catastrophic ecological consequences (Brenchley et al., 2001). Forming the vanguard of decline are the amphibians, which consist of three orders: the anura (frogs and toads), the caudata (newts and salamanders) and the gymnophiona (caecilians) (Duellman & Trueb, 1994; Zug et al., 2001). With almost one third of all species threatened with extinction and roughly 500 listed on the International Union for the Conservation of Nature’s (IUCN) Red List of Critically Endangered Species™ (IUCN, 2015), amphibians represent one of the most imperiled group of animals on the planet.

The magnitude of the current loss of species is thought to herald the sixth mass extinction in the lifetime of the planet and the biggest since the departure of the dinosaurs 65 million years ago, which, ironically, the amphibians survived (Wake & Vrendenberg, 2008). Recent research implicates an array of causal factors for population declines, and in addition to anthropogenic contributions such as habitat loss and fragmentation, other significant factors include global climate change and emerging infectious disease (Allentoft & O’ Brien, 2010; Stuart et al., 2004; Wake & Vrendenburg, 2008). As custodians of the planet, we bear a responsibility to give the protection and preservation of amphibians our highest priority and to prevent any further extinctions on our watch.
Conservation strategies for minimizing biodiversity loss include *in-situ* measures through the preservation and restoration of native habitat, and *ex-situ* measures, such as the removal of a wild species into captive assurance colonies (Hambler & Canney, 2004; Watson & Holt, 2001). Although the more desirable conservation action is certainly habitat restoration, it is not always possible due to complete habitat destruction, unmitigated threats, or political obstacles (Holt & Watson, 2001; Tudge, 1992). Consequently, many amphibian species will be eliminated before *in-situ* rescue measures can be initiated. Therefore, we must endeavor to preserve as much extant anuran diversity as possible through an *ex-situ*-based approach by promoting captive breeding and genetic management as a valuable components of the rescue efforts of declining species.

Captive assurance colonies are rapidly being established worldwide to provide a means to safeguard vulnerable species and augment populations, with the goal of future reintroductions. The overall success of reintroduction efforts, however, is contingent on the maintenance of a genetically healthy captive population through the careful genetic management of captive breeding programs. This can be somewhat challenging as amphibians are innately sensitive to environmental and endogenous stimuli as cues for breeding (Tsai, 2011). In captivity, these cues are often absent or entirely unknown and as a result, natural reproductive behaviour is often not exhibited.

Assisted reproductive technologies (ARTs) are a suite of conservation tools that have the potential to circumvent this problem and enhance captive breeding efforts *in lieu* of natural reproductive behaviour. ARTs such as exogenous hormone induction of gamete production and reproductive behaviour, artificial fertilization for population augmentation, and the long-term storage of gametes through cryopreservation, provide a
means to propagate captive populations and secure valuable genetics in perpetuity (Kouba & Vance, 2009). Conveniently, the majority of frog and toad species exploit external fertilization for reproduction meaning that ARTs such as artificial fertilization are reasonably straightforward to apply (Kouba et al., 2009). Following exogenous hormone stimulation, gametes expressed by both males and females can be collected and combined in a dish for fertilization. The resulting embryos require minimal input as they develop through metamorphosis. Therefore, in theory, it is relatively simple to maximize the fertilization rates of captive anuran species thereby boosting populations for eventual reintroduction.

However, the diverse nature of anuran reproductive modes, paramount to their evolutionary success, presents many challenges to the development of hormone protocols, which tend to be established on a species-specific basis. A major limitation on progress is the paucity in knowledge of anuran reproductive physiology, which could jeopardize ex-situ conservation efforts and threaten the success of captive breeding programs. Therefore, increasing our knowledge base of anuran endocrine function will allow us to better understand the hormonal responses that underlie reproductive behaviour in target species and develop appropriate ARTs accordingly.

The studies presented in this manuscript are based on four anuran species; one model species and three species of conservation concern. The anticipated outcomes of this research are to:

**Chapter III**: develop a successful exogenous hormone protocol for the collection of spermatozoa and extension of sperm longevity through cold-storage
techniques in the declining Southern Rocky Mountain boreal toad

(*Anaxyrus boreas boreas*).

**Chapter IV**: establish the importance of a hibernation period in captive male *A. b. boreas* through a comparative study quantifying the reproductive response to exogenous hormone treatment of hibernated versus non-hibernated male toads.

**Chapter V**: explore the potential recovery of spermatozoa from long-term cold storage by developing an effective cryopreservation protocol using the model species Fowler’s toad (*Anaxyrus fowleri*) with subsequent application to three target species: the Southern Rocky Mountain boreal toad, the critically endangered Mississippi gopher frog (*Lithobates sevosa*), and the critically endangered Puerto Rican crested toad (*Peltophryne lemur*).

**Chapter VI**: apply cryopreservation techniques to a critically-endangered Ranid species, the Mississippi gopher frog (*L. sevosa*) to ascertain the transfer potential of a successful freezing method to a different Genus. In addition, explore the feasibility of incorporating cryopreservation technology into the recovery efforts of endangered species by evaluating the fertilization capacity and developmental outcomes of frozen-thawed *L. sevosa* spermatozoa through artificial fertilization.

**Chapter VII**: validate a technique to facilitate genetic linkage of *in-situ* and *ex-situ* anuran populations through cryopreservation and cold-storage of
spermatozoa from wild-caught *A. b. boreas* and artificial fertilization using captive female gametes.

The unprecedented rate at which global anuran populations continue to decline emphasizes the urgent need to develop, refine and optimize assisted reproductive technologies to enhance captive breeding efforts. This will allow us to maximize reproductive success and ensure that the reigning survivors of previous mass extinction events can survive their own extinction crisis.
CHAPTER II
LITERATURE REVIEW

Frogs: Evolution’s great success story

There are over 7000 described species of amphibian, of which the anura (Ranidae: true frogs and Bufonidae: true toads) comprise 445 genera in 55 families (AmphibiaWeb, 2015). As survivors of four previous global mass extinction events from the Late Devonian (~364 Mya) to the Cretaceous-Tertiary boundary (~ 65 Mya), amphibians are among the oldest and most successful species on the planet (Wake and Vrendenburg, 2008). Fundamental to their evolutionary success is a vast diversity in life-history strategies that enabled exploitation of a wide variety of aquatic and terrestrial habitats (Duellman and Trueb, 1994). From their sea-dwelling ancestors to the thousands of species represented by almost every continent on earth, the persistence of these evolutionary lineages is testament to the immense success of these most fascinating creatures.

Terrestrial amphibian fossils have been uncovered from mid-Devonian sedimentary rock formations, dating back over 350 million years (Duellman and Trueb, 1994; Zug et al., 2001). From primitive amphibious creatures, further groups would evolve with a variety of aquatic, terrestrial and semi-aquatic lifestyles, giving rise to the Lissamphibia, or living amphibians (Rich et al., 1996; Zug et al., 2001). This class is represented by the three major lineages we see today: the order caudata (newts
and salamanders), the order gymnophiona (legless caecilians) and the most species-rich order anura (frogs and toads) (Duellman and Trueb, 1994) representing the greatest reproductive diversity of all tetrapod vertebrates (Crump, 2015).

**Life history and ecology**

Generally speaking, the lifecycle of an anuran is bimodal, alternating to varying degrees (depending on species) between aquatic and terrestrial environments and for the majority of species involves egg, larval, juvenile and adult stages (Duellman and Trueb, 1994; Zug et al., 2001). Anuran life-histories are considered complex due to the morphological, physiological and behavioral changes that occur during metamorphosis and changing habitat between larval and juvenile stages (Zug et al., 2001). Proximity to water is crucial as nearly all species exhibit external fertilization with aquatic larvae (tadpoles). Gills are the major respiratory structures in larvae, which are retained along with a tail before completion of metamorphosis whereby the tail regresses, lungs replace the gills, and limbs are formed (Duellman and Trueb, 1994). On emergence from the natal pond, a fully developed terrestrial anuran is well adapted for respiration by way of gas exchange through the skin, which is the major respiratory surface in adults (Duellman and Trueb, 1994; Zug et al., 2001). The highly vascularized and permeable skin of the anuran is an exceptionally dynamic organ, serving not only in respiratory function, but also in body temperature regulation, water balance, and protection (Duellman and Trueb, 1994; Zug et al., 2001).

Terrestriality places certain physiological limitations on frogs and toads and the necessity to maintain moist skin for gaseous exchange and osmoregulation makes them particularly vulnerable to desiccation and fluctuations in the environment (Duellman and
Trueb, 1994; Zug et al., 2001). Consequently, the majority of anurans are limited by their physiology and, as a result, the most species-rich areas are the neo-tropical countries, accounting for around 80% of global frog biodiversity (IUCN, 2015). Nevertheless, despite a physiology that seemingly might restrict global anuran distribution to a small variety of habitats, frogs and toads have evolved a remarkable array of physiological, morphological and reproductive strategies to survive life in almost all ecoregions, except for the oceans, and every continent with the exception of Antarctica (Duellman and Trueb, 1994; Pinder et al., 1992). Frog and toad species inhabiting regions of unfavorable conditions are able to condense their life cycle into intermittent periods of breeding and feeding activity when environmental conditions have improved (Pinder et al., 1992).

**Hibernation and Estivation**

Anuran species that live in extreme environments face a number of challenges and a period of dormancy is a common strategy for survival (Storey and Storey, 1992). Hibernation provides a means to circumvent limited food availability and reduce the energetic costs of maintaining body temperature during seasonally cold periods (Storey, 2000). In order to survive at sub-zero temperatures, there are three basic methods of overwintering for northern anuran species (1) terrestrial hibernation in underground hibernacula, (2) freeze-tolerant terrestrial hibernation, or (3) submergence under water (often under ice cover) (Pinder et al., 1992; Storey, 2000).

Terrestrial hibernation in most northern climate anurans is via avoidance in a hibernacula under the frost line, as exhibited by a number of Bufonidae including the boreal toad (*Anaxyrus boreas boreas*) (Pinder et al., 1992; Zug et al., 2001). The
alternative, and rather more extreme, method of terrestrial hibernation is freeze-tolerance whereby a small number of anuran species can hibernate at the soil surface and survive periods of supercooling at temperatures as low as -8°C (Storey and Storey, 1992; Wells, 2007; Zug et al., 2001). The initiation of ice formation triggers the production and accumulation of cryoprotectants, raising tissue levels of either glucose (as in *Pseudacris crucifer, P. triseriata* and *Rana sylvatica*), or glycerol levels (as in *Hyla versicolor*) to depress the freezing point of bodily fluids and prevent intracellular ice formation (Layne and Kefauver, 1997; Storey, 2000; Wells, 2007; Zug et al., 2001). As the reproductive season approaches and temperatures climb, the cryoprotectants are slowly drawn out of the cells as the frogs thaw (Storey, 2000). Aquatic hibernators are mostly frog species and avoid harsh environments by hibernating in the oxygen-rich water column at the bottom of ponds where high-density water does not drop below 4 °C (Pinder et al., 1992; Zug et al., 2001).

Estivation is a survival strategy employed by anuran species to exploit the other extreme of the temperature spectrum. Frogs and toads that dwell in seasonally arid environments enter a torpid state that allows them to endure long periods of heat and lack of food (Corn, 2005; Zug et al., 2001). Both hibernation and estivation have in common a retreat into a sheltered location, typically underground in soil, sand or mud (Storey, 2000). Some species can spend as many as 10 months burrowed underground emerging only to feed and breed when environmental conditions have improved (Storey and Storey, 1992).
Reproductive strategies

Wells (1977) divided anuran reproduction into two primary modes: explosive breeding and prolonged breeding. Both systems are found within the Bufonidae and the Ranidae, however generalizations can be made within the toads which tend to be explosive breeders and the Hylidae (treefrogs) which are generally prolonged breeders. These two strategies represent a spectrum of reproductive characteristics ranging from reproduction in large congregations occurring in a single night to reproduction over several months (Wells, 1977). Hormonal controls mediate reproductive timing, but ultimately reproduction in the anurans is triggered directly or indirectly by environmental cues such as temperature rainfall or photoperiod (Zug et al., 2001). Reproduction varies depending on seasonality and among the anura there are highly seasonal species, such as the majority of temperate zone anurans (Zug et al., 2001), and tropical species, the majority of which tend to be aseasonal breeders (meaning continuous or opportunistic mating) (Zug et al., 2001). A major factor in reproductive strategies is the predictability of the environment relative to each stage of the life history. Generally speaking, anurans inhabiting stable environments tend to follow the general pattern of (1) late maturity, (2), multiple clutches, (3) fewer but larger eggs, (4) parental care, and (5) small reproductive efforts favored (Duellman and Trueb, 1994). In contrast, in fluctuating environments with uncertain larval survivorship the opposites should be followed (Duellman and Trueb, 1994; Crump, 1982).

The widespread distribution of frog and toad species in a variety of habitats corresponds to a tremendous diversity in reproductive strategies. Indeed, anurans have the highest diversity in reproductive strategies of all terrestrial vertebrates and this
dictates a similar diversity in the physiological control of reproduction, including hormonal control of sexual behavior, spermiation, spawning, and oviposition (Duellman and Trueb, 1994; Tsai, 2011). A ‘reproductive strategy’ relates to a set of adaptations that have, on average, contributed to the greatest average number of offspring to recent generations (Duellman and Trueb, 1994). There are some 39 documented modes of reproduction for the anurans, ranging from aquatic egg and larvae to viviparity and direct development (Crump, 2015; Duellman and Trueb, 1994) and, despite some commonalities with regards reproductive patterns, no generalizations can be made about amphibian reproductive modes (Duellman and Trueb, 1994). Anuran reproductive strategies are defined by a combination of characteristics including: the method of fertilization (internal or external); location of egg deposition (terrestrial or aquatic); clutch structure; larval development site; the rate and duration of embryonic development; and levels of parental care (if any) (Duellman and Trueb, 1994). This variety of characters corresponds to equally diverse life histories and reproductive patterns within the anurans (Zug et al., 2001). Reproductive modes range from the more primitive aquatic-terrestrial mode of external fertilization where the general manner of copulation requires a male and female pair to engage in a mating clasp (amplexus) for cloacal alignment (Duellman and Trueb, 1994). With only a couple of exceptions, male anurans lacks an intromittent organ, therefore cloacal alignment is key to maximizing fertilization rates as sperm is released onto the eggs protruding from the female cloaca (Kuhnel et al., 2010). In rare cases, fertilization can take place without amplexus whereby a male sits on previously deposited eggs and releases spermic urine (Crump, 2015). Generally speaking, with aquatic externally-fertilizing species, eggs are left to
develop unattended and aquatic larvae will hatch into the surrounding water to complete metamorphosis to juvenile stage in the absence of any parental care (Duellman and Trueb, 1994). To survive and grow, the embryos of frogs and toads must have a sufficient supply of nutrients which in the majority of species that exhibit oviparity, is provided by a yolk within the egg (Zug et al., 2001).

However, parental care is not entirely lacking across the externally-fertilizing anurans and some 6% of species exhibit a number of diverse approaches (Crump, 1996; Duellman and Trueb, 1994) including: tadpole transport from terrestrial oviposition sites to water bodies (e.g. male Mallorcan midwife toad; Alytes obstetricans), transportation of eggs, either in the vocal sac (e.g. the male Darwin’s frog; Rhinoderma darwinii), or in pouches or depressions on the back (e.g. female Suriname toad; Pipa pipa) (Crump, 1996; Duellman and Trueb, 1994; Wells, 2007). The mountain chicken frog (Leptodactylus fallax) exhibits a high level of parental care by producing infertile eggs to feed newly hatched tadpoles, a trait which is also displayed by a number of other species (Duellman and Trueb, 1994). Perhaps the most dedicated example of parental care in the anurans belonged to the now-extinct, gastric-brooding frog (Rheobatrachus silus). Female R. silus suppressed their digestive system to enable incubation of swallowed fertilized egg masses in the stomach before regurgitation of the fully developed offspring 7-8 weeks later (Tyler et al., 1983).

A major advancement towards terrestiality in the evolution of the anurans was the deposition of larger-yolked eggs out-with an aquatic environment (Crump, 1996). This mode is represented by a number of anuran species, both direct and indirect developers and species exhibiting internal fertilization and the capacity to bear live
young; although the latter two modes are rare (Duellman and Trueb, 1994; Wake, 1993; Zug et al., 2001). Various sites of egg deposition include: moist environments with close proximity to a water body (Haddad and Prado, 2005), on vegetation suspended above water (Gomez-Mestre et al., 2006), and securing eggs within a foam nest, either floating on the water e.g. or in shallow burrows (Downie, 1990). The desert rain frog (*Breviceps macrops*) is a terrestrial breeder and produces direct-developing offspring, which emerge fully formed from eggs burrowed in the ground, completely circumventing the aquatic larval stage (Channing and Whalberg, 2011).

Internal fertilization is thought to have evolved a number of times but is known in only a few species of frog (Kuhnel et al., 2010; McDiarmid and Altig, 1999) and is generally considered a prerequisite for viviparity, which is associated with more advanced levels of parental care (Wake, 1993). Reproductive modes of internally-fertilizing anuran species are also diverse and can be categorized based on site of egg deposition, and tendency towards terrestriality by the production of larger yolks and directly-developing young (Duellman and Trueb, 1994). For example, the tailed-frog (*Ascaphus truii*) achieves reproduction in a fast-flowing river habitat via an intromittent-like organ. The ‘tail’ is in fact an extension of the cloaca and delivers sperm directly into the female to prevent the loss of gametes in surrounding water and the female gives birth to live froglets (Broan, 1972). In three of the species exhibiting retention of eggs within the oviduct - *Nectophrynoides torneri*, *N. viviparus* and (possibly-extinct) *Eleutherodactylus jasperi* – the eggs develop with nutrition provided by the yolk before live birth, and hence are ovoviviparous (Duellman and Trueb, 1994). However, *N. liberiensis* and *N occidentals* exhibit true viviparity whereby maternal nutrition is
provided by oviductal secretions with the subsequent birth of froglets (Duellman and Trueb, 1994; Wells 2007). A recently described novel reproductive strategy is that of the Asian fanged frog (*Limnonecectes larvaepartus*) which is unique among the anurans in having both internal fertilization and birth of live tadpoles (Iskandar et al., 2014). The fact that novel reproductive modes continue to be described in the anurans highlights the sheer challenge of ART in the amphibians.

**Amphibian reproductive system**

Until recently, little was known about the form and function of the neurohormones regulating amphibian reproduction. However, several studies indicate that the neuroendocrine basis of the anuran reproductive system is highly conserved (Rastogi *et al*., 2011; Tsai, 2011) and the synthesis and release of neurohormones emulates the classic endocrine circuitry of other vertebrates (Licht and Porter, 1987; Peter, 1983; Rastogi *et al*., 2011; Wright and Whitaker, 2001). The last several decades have yielded a number of physiological studies indicating that neurohormones secreted by the hypothalamus convey environmental stimuli to peripheral tissues for reproductive regulation through the actions of a hypothalamic-pituitary-gonadal (HPG) axis (Tsai, 2011).

**The Hypothalamic-Gonadal-Pituitary (HPG) axis**

Due to their vast diversity in reproductive modes, anurans are ideal model systems in which to investigate and clarify the role of neurohormones (steroid hormones, brain and pituitary hormones) in the maintenance and regulation of external-environment-(HPG) axis (Rastogi *et al*., 2011). In response to favorable environmental conditions, a
hormonal cascade is initiated whereby neurohormones released by the hypothalamus stimulate the pituitary release of glycoprotein hormones that regulate gonadal activity (Tsai, 2011). Mature gonads produce steroid hormones that stimulate steroidogenesis in the testes, follicular growth and ovulation in the ovaries, and yolk formation (vitellogenesis) in the liver (Rastogi et al., 2011). There are indications that the steroid hormones are under both positive and negative feedback control for the upregulation or downregulation of neurohormone secretion by the hypothalamus (Tsai et al., 2005) (Figure 1).
The Hypothalamic-pituitary-Gonadal (HPG) axis featuring hormone cascade events. GnRH is released by the hypothalamus (HYP) in response to environmental or endogenous cues and binds receptors at the anterior pituitary (AP). The gonadotropin hormones Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) are released from the AP. LH and FSH act at the level of the gonads to stimulate steroidogenesis in the testes, and follicular growth and ovulation in the ovaries and yolk formation (vitellogenesis) in the liver. LH is the regulator of the Leydig cells which produce testosterone (T) and estrogens (E). LH secretion is under the negative feedback regulation of testicular steroids that act to suppress GnRH secretion from the HYP. Testosterone produced by the Leydig cells is transported to Sertoli cells where it is converted into dihydrotestosterone (DHT) and estrogen which are thought to exist as both positive and negative feedback mechanisms among anuran species.
The gonadotropin-releasing hormone systems

The hypothalamic decapetide gonadotropin-releasing hormone (GnRH) is likely highly conserved across the vertebrates (Rastogi et al., 2011; Tsai, 2001). GnRH stimulates the synthesis and pituitary release of the gonadotropin hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that regulate downstream gonadal effects (Licht and Porter, 1987; Zerani et al., 1991). Brain lesion experiments in anuran model species elucidated the significance of GnRH in the anurans and confirmed its presence in two discrete regions of the brain (Licht and Porter, 1987; Peter, 1983). The regulation of pituitary gonadotropin activity by the GnRH system in anurans is now generally recognized as homologous to that of mammals, as well as other vertebrates, including mammalians, chickens and salmon (Licht and Porter, 1987; Licht and McCreery, 1985). Like mammals, it is believed that anurans possess two distinct GnRH systems in the brain: GnRH-I and GnRH-II (Fernald and White, 1999; Tsai, 2011). GnRH-I is physiochemically and immunologically similar to that of mammalians and is the predominant stimulatory form in the amphibian brain (Daniels and Licht, 1980). However it remains unknown whether or not GnRH-II, which is analogous to salmonid GnRH, also participates in neuroendocrine regulation of the anterior pituitary (Tsai, 2011).

Modulation of the GnRH system by dopamine

The central dopaminergic system is an important inhibitory component in the regulatory circuitry of brain GnRH levels in a variety of taxa (Dufour et al., 2005; Tsai, 2011). The secretion of the catecholamine dopamine (DA) neurotransmitter is a strong inhibitor of LH release (Tsai, 2001). Studies in fish confirm the presence of an inhibitory
neuroendocrine mechanism controlling gonadotropin secretion by inhibitory dopamine neurons (Dufour et al., 2005; Trudeau, 1997). However, there is limited evidence that an LH-inhibitory system exists in amphibians (Tsai, 2011). Preliminary brain lesion experiments in frogs showed that a DA antagonist elevated GnRH levels, leading to elevated LH and ovulation (Sotowska et al., 1994). Further exploration of the neuroendocrine control of dopamine on amphibian reproduction is necessary.

Gonadotropin hormones

The gonadotropin hormones LH and FSH are responsible for conveying environmental stimuli to peripheral tissues for ovulation and sperm release (spermiation) (Licht and Porter, 1987; Tsai, 2011). Originally, it was thought that anurans may possess only one gonadotropin responsible for the range of LH- and FSH-related actions (Licht and McCreery, 1985). However, two distinct gonadotropins that structurally and functionally resemble mammalian FSH and LH were identified in several anuran species (Licht and Porter, 1987). Pituitary release of FSH and LH induces the production of androgens (testosterone) in males and estrogens (estradiol) in females (Rastogi et al., 2011). LH acts at the level of the gonads to stimulate steroidogenesis in the testes and follicular growth, ovulation in the ovaries and vitellogenesis (yolk formation) in the liver and is under negative feedback regulation of testicular steroids that act appear to suppress GnRH secretion from the hypothalamus (Rastogi et al., 2011). In males LH receptors are located in the Leydig cells where LH controls the production and release of testosterone and estrogens and it is also responsible for hydration of the Sertoli cells (Burgos and Vitale-Calpe, 1967). In the Sertoli cells testosterone is transported and converted into
dihydrotestosterone (DHT) and estrogens (E) which are feedback regulators of
gonadotropin secretion by the pituitary in many frog species (Pavgi and light, 1993).

Many studies suggest that androgens play an important role in spermatogenesis
and spermiogenesis, indicating that they are important both in the mitotic and meiotic
phases of sperm production in the amphibians (Propper, 2011; Raucci and Di Fiore,
2007). The hypothesized role of FSH is predominantly during the initial stages of the
reproductive cycle by mediating germ cell recruitment, mitosis and meiosis and it is also
thought to be effective at inducing spermiation (Propper, 2011; Rastogi et al., 2001).
In females LH- receptors are located on the theca and granulosa cells and provide
androgens and hormonal precursors for estradiol production to promote egg maturation,
ovulation and spawning (Clulow et al., 2014). Ovarian follicles, under the influence of
pituitary gonadotropins secrete estradiol, which is required to stimulate the synthesis of the
yolk precursor protein vitellogenin by the liver (Rastogi et al., 2011). When favorable
conditions for spawning occur, the pituitary releases LH and FSH and oocytes move toward
final maturation (Rastogi et al., 2011). Estradiol secretion diminishes during this time and
under the influence of FSH the follicles secrete progesterone, promoting the final
maturation of oocytes (Rastogi et al., 2011).

There is evidence to suggest that DHT and E provide both positive and negative
feedback, respectively, on gonadotropin secretion at the level of the anterior pituitary and
hypothalamus (Pavgi and light, 1993). Upregulation of FSH and LH by positive feedback
of DHT and E also been reported in some frogs (e.g. Lithobates. pipiens and Rana
esculenta, respectively) underlining the premise that generalizations about endocrine
system function in the anurans cannot necessarily be made (Tsai et al, 2005). With
limited information available, it is important to trial homologous gonadotropins in more species to elucidate the underlying mechanisms of LH and FSH synthesis and release in amphibians (Clulow et al., 2014; Propper, 2011).

**Testes and spermatogenesis**

The spermatogenic cycle is completed in the testes, which in anurans are anatomically characterized as single bilateral ovoid structures, organized in tubules (Duellman and Trueb, 1994; Tsai, 2011). Within the testes are cyst-filled testicular lobules from which sperm is released through efferent ductules to the kidney (Tsai, 2011). The ducts (vas efferentia) from each testis pass along the mesorchium and lead to the Wolffian or mesonephric duct (ureter) that transports urine and sperm to the cloacal for expulsion (Duellman and Trueb, 1994; Mobjerg et al., 2000).

Spermatogenesis occurs in cysts composed of Sertoli cells enveloping germ cells at synchronous stages (Duellman and Trueb, 1994). Testicular sperm are embedded in the Sertoli cells until gonadotropin-induced hydration to expel spermatozoa into the seminiferous tubules (Burgos and Vitale-Calpe, 1967). LH, released by the pituitary, targets the cytoplasm of the Sertoli cells. Water, which enters the channels of the endoplasmic reticulum, is attracted to the Sertoli cells by hydrophilic forces, causing them to burst and release spermatozoa into the lumen of the seminiferous tubules (Burgos and Vitale-Calpe, 1967). In continuously breeding species of frogs and toads, such as tropical species, the testes contains sperm cells at varying stages of development and a lobule may contain cells ranging from primary spermatogonia to mature sperm cells (Duellman and Trueb, 1994; Tsai, 2011). Whereas in discontinuous breeders, such as temperate anuran species, spermatogenesis occurs during or directly following the
breeding season and stored in seminiferous tubules until the initiation of breeding when sperm cells mature homogenously throughout the testes, prior to release in urine (Duellman and Trueb, 1994).

Gonadotropins act on the testis via receptors, but the response of the gonads depends on local mechanisms (Fasano and Pierantoni, 1993). The presence of various cell types within the same compartment requires coordination of cell functions to facilitate the production of spermatozoa (Pierantoni et al., 2002). There is evidence of cellular communications via paracrine/autocrine mechanisms in the testis of anurans but the underlying mechanisms are not yet fully understood (Pierantoni et al., 2002).

Spermatozoon are highly modified cells with three sections: a head, a mid-piece containing mitochondria and a tail (Zug et al., 2001). The Bufonidae have an accessory cell on the spermatozoa, called a mitochondrial vesicle (MV) that contains a high concentration of mitochondria and also appears to contain a nucleus (Roth and Obringer 2003; Waggener and Carroll, 1998). The presence of the MV is thought to be related to motility and reduced motility capacity has been associated with MV damage (Kouba et al., 2003).

Ovaries and ovulation

The amphibian ovary contains two types of oocytes: small gonadotropin-independent oocytes and larger gonadotropin-dependent oocytes (Wright and Whitaker, 2001). Across all reproductive modes in the anurans, the female reproductive cycle is characterized by a vitellogenic phase of ovarian growth which is preceded by the recruitment of previtellogenic follicles (Rastogi et al, 2011). The anuran ovarian cycle can be divided into three distinct stages: a pre-spawning phase, an ovulatory phase, and a
postovulatory stage (Rastogi et al., 2011; Wright and Whitaker, 2011). The ovarian follicles, under the influence of pituitary gonadotropins, secrete estradiol, which is required to stimulate the synthesis of the yolk precursor protein vitellogenin by the liver (Rastogi et al., 2011). The majority of the process whereby the yolk is synthesized and incorporated into the oocyte (vitellogenesis) takes place during the pre-spawning phase (Rastogi et al., 2011; Wright and Whitaker, 2001). When favorable conditions for spawning occur oocytes move toward the ovulatory or spawning phase during which the follicles secrete progesterone which promotes final maturation of oocytes (Rastogi et al., 2011). Several jelly layers surrounding the oocyte are synthesized and deposited on the egg as it passes through the oviduct and the eggs are maintained in the female’s ovisac until the appropriate conditions for spawning (Wright and Whitaker, 2001).

Many tropical species exhibit prolonged breeding with asynchronous oogenesis whereby the ovary contains multiple stages of follicular growth throughout the year so as to maintain a readiness to breed when favorable conditions arise (Rastogi et al., 2011). In contrast, in temperate female anurans (Rastogi et al., 2011), characterized by explosive breeding, there is synchrony of vitellogenic oocytes (Rastogi et al., 2011). The anurans are the most fecund of the amphibians, laying between a few hundred to a few thousand small yolked eggs between 1 – 2 mm in diameter (Rastogi et al., 2011).

**Anuran fertilization**

Generally speaking, the anuran exhibit external fertilization and sperm are stored in a quiescent state in the male reproductive tract until the decrease in osmolality with spermiation activates sperm motility (Duellman and Trueb, 1994; O’ Brien et al., 2011). The underlying signaling mechanisms of the pathway is mostly unknown, although there
is strong evidence towards the role of cAMP-mediated Protein Kinase A in the activation of sperm motility (O’ Brien et al., 2011). Immediately upon spawning, several jelly layers surrounding the oocyte release molecules that result in a hypotonic fertilization media for sperm activation (Krapf et al., 2007; O’ Brien et al., 2011). Spermatozoa must pass through the jelly layers that surround the oocyte in order to achieve fertilization (O’ Brien et al., 2011). The passage of sperm through the egg jelly layers is thought to emulate the capacitation stage of mammalian fertilization and take place before the acrosome reaction (AR) (Krapf et al., 2007; Shiver and James, 1970). Anuran sperm have an acrosome containing a vitelline lysin on the periphery of the head and the AR appears to be performed in species-specific location of the egg (Ueda et al., 2002). In the Mediterranean painted frog (Discoglossus pictus), the AR is triggered in the jelly layers of the egg whereas it occurs on the vitelline envelope (VE) in the Japanese common toad toads (Bufo Japonicas) (Ueda et al., 2002) and the Argentine common toad (Bufo arenarum) (Krapf et al., 2007). Sperm-egg binding on the VE appears to require a homolog of the mammalian sperm receptor ZP2 and the AR in Xenopus laevis is similar to mammals in that it comprises exposure of the inner acrosomal membrane without formation of a prominent acrosomal process (Ueda et al., 2002). The mechanisms of induction of the acrosome reaction and its importance in the fertilization process of the anura have not yet been elucidated.

The Amphibian Extinction Crisis

Several decades ago, field biologists in a number of locations around the world began to observe a strange phenomenon; entire populations of amphibians were disappearing from places they were historically common (Crump and Pounds, 1994;
Moore, 2014; Zug et al., 2001). Disconcerting as these anomalies were, their significance would not be acknowledged by the global conservation community for several decades to come (Wake, 2001). What field biologists at the time didn’t know was that they were witnessing what might be the greatest biodiversity crisis in the history of humanity (Bishop, et al., 2012) in which amphibians would play an increasingly conspicuous role (Beebee and Griffith, 2005; Blaustein and Bancroft, 2007; Stuart et al, 2004).

A predicted thirty to fifty species from a variety of taxa are heading toward extinction within the next quarter of a century (Thomas, et al., 2004). With around 135 species believed to have gone extinct in the last three decades alone (McCallum, 2007), amphibians likely hold the unfortunate title of most threatened vertebrate group (Stuart et al., 2004). Recent estimates indicate that over forty-percent of amphibian species are considered threatened or endangered (IUCN, 2015). More troubling still, is the likelihood that this number is much higher if all undescribed amphibian species are included (Stuart et al., 2004). The five known previous mass extinctions and documented extinction rates for sink populations within metapopulations, which can be measured in a few generations, illustrate that such mass extinction events relative to geological time, and local extinctions in ecological time, are a natural part of life on Earth (Blaustein et al., 1994; McKinney, 1997). However, in contrast to the typical rates of tens of thousands to millions of years for a species to become extinct (Zug et al., 2001), current extinction rates are an estimated 1,000 to 10,000 times natural background rates (Chivien and Bernstein, 2008; Pimm et al., 2014), which puts the extinction rate of amphibians at 25,000 to 45,000 times typical rates (McCallum, 2007). Growing evidence suggests that we are in the midst of a mass extinction crisis (Leakey and Lewin, 1995; Wake and
Vrendenburg, 2008). The sixth in the lifetime of the planet. The first in the history of humanity.

**Global forces of destruction**

Unlike previous mass extinctions that were caused by major catastrophic events such as asteroid strikes and volcanic eruptions, the current loss of animal and plant species are largely anthropogenic in origin (Becker et al., 2007). Habitat loss and fragmentation likely play a significant role in amphibian declines, although, other factors, such as ultraviolet radiation, pollution, global climate change and emerging infectious disease, are also implicated as contributing factors (Blaustein et al., 2004; Beebee and Griffiths, 2005; Stuart et al., 2004; Wake and Vrendenburg, 2008). The general consensus is that there is no single cause for such dramatic declines, rather multiple factors acting synergistically (Blaustein and Bancroft, 2007). More alarmingly still, are the amphibian declines being documented in pristine areas (so-called enigmatic declines) undisturbed by humans, such as national parks, conservation zones and remote locations far removed from any urban areas (Blaustein et al., 1994). In recent decades, infectious disease has impacted amphibians worldwide, largely a result of the fungal disease chytridiomycosis (*Batrachochytrium dendrobatidis*; Bd) which has contributed to rapid global population crashes (Berger et al., 1998; Gascon et al., 2007; Skerratt et al., 2007).

**The importance of frogs**

*The ecological role of frogs*

Anurans play a significant, if unsung, role in maintaining the fine ecological balance of the planet and, therefore, warrant considerable conservation attention (Reaser
and Blaustein, 2005). Frogs are important bioindicator species, vulnerable to environmental perturbations in both aquatic and terrestrial ecosystems, due to the permeability of their skin and amphibious lifestyle (Duellman and Trueb, 1994). A revival of interest in amphibian declines in the late 1980s prompted a wave of ecotoxicological studies on frogs and toads (Simon et al., 2011) and their use as bioindicators of contaminant accumulation in pollution studies is rapidly increasing (Johansson et al., 2001). Anurans have multiple functional roles in both terrestrial and aquatic ecosystems, and their contribution to biodiversity and integral intermediary role in the food web means that local frog population declines can affect overall ecosystem stability (Reaser and Blaustein, 2005). Aquatic larvae play a vital ecological role in freshwater system stability due to the diversity in their functional groups, with relative ecological importance (i.e. carnivore, herbivore, detritivore, and filter or suspension feeding larvae). Loss of tadpoles in aquatic waterways would have significant impacts on algae assemblage, other aquatic fauna and overall water quality (Duellman and Trueb, 1994; Ranavestel et al., 2004). In addition, frogs and their larvae influence food web dynamics and energy flow and are an important source of prey for a number of reptiles, birds and small mammals and, in turn, are key natural pest-control agents of human and agricultural disease vectors, as well as other insects and worms (Duellman and Trueb, 1994). Frogs and toads are an important link between humans and ecosystem health and their disappearance is a warning sign about the health of the wider habitat (Hayes et al., 2002), which we ignore at our peril.
Nature’s pharmacy

Frog skin secretions have been an area of particular interest to scientists in recent years, and their potential application to improving human health has delivered some promising findings. The natural pathogen defense system in frog skin is an abundant source of peptides with broad spectrum antimicrobial activity against a host of bacteria, viruses and fungi (Woodhams et al., 2014). Since most human infections occur across a mucous membrane, the therapeutic potential of these peptides is being explored as a possible source of longer-lasting and, theoretically, more effective antibiotic agents (Woodhams et al., 2014). The development of novel antibiotics would be a major pharmaceutical coup in the fight against growing global resistance to multiple conventional therapies. There is a suite of other potential uses from the antimicrobial peptides on frog skin, such as the discovery of an analgesic more effective at relieving pain than morphine and void of many of the undesirable side-effects (Daley et al., 2003). This new compound (EBT-594) is sourced from the toxin of a deadly South American poison dart frog and could be developed into a new drug to provide relief for the estimated 40 million chronic pain sufferers in the U.S alone (Daley et al., 2003). Proteins in the secretions of the waxy monkey frog (Phyllomedusa sauvagii) and giant fire-bellied toad (Bombina orientalis) have been proposed as potential therapies for more than seventy major diseases, including diabetes and cancer (CORDIS, 2009). To date, the chemical structure of these antimicrobial peptides has been determined for only 200 species of frogs and with a further 6000 still awaiting analysis, there is enormous potential for further significant discoveries (Conlon, 2004).
However, despite the encouraging recent momentum in research, many aspects of frog chemical ecology remain unexplored and there is a sense of urgency in unlocking the potential of the natural world before it is too late. The many significant pharmaceutical developments sourced from frog species highlights the importance of this research and emphasizes the significance of preserving extant biodiversity. Sadly, there are extinct frogs from which we are no longer able to gain knowledge, such as the gastric brooding frog species (*Rheobatrachus spp.*) and the potential to provide treatment for the more than 25 million people suffering from peptic ulcers (Tyler *et al.*, 1983). *Rheobatrachus spp.* were discovered in Australia in the 1980s and fascinated researchers with their unique ability to gestate their young in their stomach until hatching. *Rheobatrachus spp.* were able to accomplish this feat by regulating the release of stomach acids and enzymes so as not to digest their young (Hutchinson and Maxson, 1987). Analysis of these compounds was in the preliminary stages when the gastric brooding frog was declared extinct in 1983; human activity was cited as the primary cause (Tyler *et al.*, 1983). Wild species possess a veritable ‘frog pharmacy’ with the potential to alleviate human suffering. When a frog becomes extinct the potential applications for improving human health also disappear.

**Global response to amphibian declines**

Preliminary reports of amphibian declines began circulating in the 1950s, however, the extent of the problem was not realized until three decades later during discussions at the 1st World Congress of Herpetology in 1989 (Beebee and Griffiths, 2005; Bishop *et al.*, 2012). Following this, the Declining Amphibian Populations Task Force (DAPTF) was established in 1991 by the Survival Commission (SSC; IUCN, 2012).
Gland, Switzerland) of the IUCN. This international conservation team of over 3,000 scientists were tasked with establishing an Amphibian Conservation Action Plan (ACAP) to ‘determine the nature, extent and causes of declines of amphibians throughout the world, and to promote means by which declines can be halted or reversed’ (Bishop et al., 2012; Gascon et al., 2007). In 1997 the DAPTF confirmed fears of a significant decline in global amphibian populations; the problem was real and serious (Heyer, 1997).

This galvanized the research community and a decade of extensive investigation later resulted in a proliferation of studies and publications, and prompted the initiation of the first Global Amphibian Assessment (GAA) by the IUCN (2004) (Bishop et al., 2012; Gascon et al., 2007; Stuart et al., 2004). The GAA involved more than 500 scientists from over 60 countries and represented the first ever comprehensive evaluation of the conservation status of global amphibian species (Bishop et al., 2012). The assessment concluded that in excess of thirty-two percent of described species were under threat of extinction at the time (IUCN, Conservation International, and NatureServe 2006). The GAA brought to the attention of the amphibian community the need to approach the crisis by means other than regular conservation strategies, which were deemed insufficient to counter the threats (Gascon et al., 2007). Moreover, it provided the impetus for further meetings, including the Amphibian Conservation Summit (ACS) in 2005 where in addition to novel threats like disease, global warming and environmental contamination, the wider issues of land degradation and fragmentation, land use change and invasive species were discussed (Gascon et al., 2007). The ACS meeting resulted in a comprehensive ACAP report, published in 2007, and the general agreement that a single solution could not alleviate or reverse the extinction crisis, rather a global multi-
disciplinary approach was necessary (Gascon et al., 2007). Furthermore, the GAA garnered considerable media coverage and brought the issue to the attention of the wider international scientific community as well as business corporations, governments and civil services (Gascon, et al., 2007)

Significant efforts within the framework of the ACAP, to establish survival assurance colonies, investigate infectious disease, and explore habitat protection measures were considered insufficient to alleviate the crisis and species continued to decline and go extinct (Bishop et al., 2012; Gascon, et al., 2007). In 2006, the Amphibian Ark (AA), an association of research institutions, captive breeding facilities and non-profit organizations, was established to review the estimated number of species in need of ex-situ conservation measures in captive assurance colonies (Bishop et al., 2012). The final tally amounted to an estimated 950 species in need of assistance, if data deficient species were taken into consideration (Bishop et al., 2012). Global captive populations currently hold only 20% of all endangered or critically endangered frog species (IUCN, 2015). Putting these figures in perspective of the total global capacity for managing viable captive breeding populations (CBPs), which is around 50 species, highlights the sheer enormity of the amphibian crisis and deficit in resources (Bishop et al., 2012). In 2011, the Amphibian Survival Alliance (ASA) formulated a global partnership to readdress key concepts, develop a global network for the implementation of the ACAP framework, and provide a more comprehensive and coordinated response to the continued loss of amphibian populations worldwide (Bishop et al., 2012).
**Ex-situ Conservation**

Although the most desirable conservation strategy is the preservation or restoration of habitat, in some cases this is simply not possible where *in-situ* threats have not been mitigated, the habitat simply no longer exists or there is a lack of cooperation between landowners or governments and conservationists (Tudge, 1992; Watson and Holt, 2001). Many species are likely to become extinct before any habitat rescue measures can be implemented (Hambler and Canney, 2004). Consequently, increasing resources are being dedicated to captive breeding and other *ex-situ* methods of conservation for species incapable of surviving in their natural habitat (Hambler and Canney, 2004; Frankham *et al.*, 2010). *Ex-situ* conservation is advantageous to species recovery in a number of ways including (1) safeguarding vulnerable populations against threats in the wild, (2) providing a tangible means to educate the wider public on the importance of conservation issues, (3) promoting species of conservation concern to raise awareness and support fundraising efforts, (4) providing a population of animals for research purposes in order to gain a better understanding of basic species biology for application to wild counterparts, and (5) augmenting declining populations and producing offspring for reintroductions (Frankham *et al.*, 2010). There are nearly 100 developing *ex-situ* programs for priority amphibian rescue species currently monitored by the Amphibian Ark, with many more being initiated in countries around the world (Amphibian Ark, 2015).

**Captive breeding programs**

Captive breeding programs (CBPs) generally involve the maintenance of the genetic diversity of a species, whilst maximizing reproductive output and avoiding the
negative effects of inbreeding (Watson and Holt, 2001) with the ultimate goal of reintroduction into the wild. Of the thirty-three animals classified as ‘extinct in the wild’ under the IUCN Red List of Critically Endangered Species™, thirty-one are actively bred through CBPs at zoological institutions, six of which currently have reintroduction programmes in place (IUCN, 2015; WAZA, 2015). CBPs and subsequent reintroductions are vital and often successful conservation tools for species already eliminated in the wild, offering the only alternative to extinction for species such as the Przewalski’s horse (*Equus ferus przewalskii*), the black-footed ferret (*Mustela nigripes*), the Californian condor (*Gymnogyps californianus*), and the Arabian oryx (*Oryx leucoryx*) (Primack, 1998; Ryder, 2000’ WAZA, 2015). Furthermore, conservation breeding is estimated to have played a role in the successful recovery of one-quarter of vertebrate species whose threat status was reduced according to the IUCN Red List of Endangered Species™ (IUCN, 2015; WAZA, 2015).

Despite the definitive evidence supporting captive breeding as one of the most essential and applicable ways to subdue a conservation crisis, it is not wholly supported as a legitimate conservation endeavor. Indeed, sceptics argue against investing in a strategy that can realistically save only a handful of species, instead of devoting resources to habitat restoration, which has the potential to conserve a wider range of fauna (Pritchard *et al.*, 1995). As such, the value of captive breeding as a conservation tool remains a source of contention (Griffiths and Pavajeau, 2008). CBPs should be promoted as an intermediary measure to protect a species and avoid extinction by linking *ex-situ* programs with measures to reintroduce them back into their native habitat as soon as is feasibly possible (Amphibian Ark, 2015). Therefore the concept of modern zoos must
be revitalized as valuable conservation research facilities by highlighting species rescue success stories and fostering effective collaborations between zoological institutions for integrating field and captive research (Burghart and Milostan, 1995). In recent years, zoos have encompassed a more considerate role in society by endorsing conservation and research as a principal aim (Hambler and Canney, 2004) and are now over 200 Association of Zoos and Aquariums (AZA) accredited zoos worldwide, many of which provide an important reservoir of endangered species that are maintained and managed through captive breeding programs (WAZA; 2014).

**Captive breeding challenges**

Captive breeding is, more often than not, employed only when other means of rescuing a species have been exhausted (Pritchard, 1995). Consequently, CBPs tend to become established when populations sizes have dwindled or a species has become exceptionally rare (Frankham et al., 2010). This creates many challenges for the management of a CBP, in addition to compromising the free-living population by removal of founder individuals (Burghart and Milostan, 1995). Fundamental to the overall success of a CBP is the careful management and maintenance of genetic diversity, which is particularly important with regards small and declining populations (Frankham et al., 2010). As gene flow becomes restricted over time, reduced allelic diversity and heterozygosity can promote inbreeding depression and an accumulation of deleterious mutations, thus lowering the survival rate of inbred individuals (Boakes et al., 2007; Ficetola et al., 2011; Taylor, 2003). This further reduces the population size and increases its susceptibility to stochastic processes (Ficetola et al., 2011; Frankham et al., 2010). Furthermore, the longer a population remains in captivity, the more liable it becomes to
genetic adaptation through selective forces (Frankham, *et al.*, 2010; Griffiths and Pavajeau, 2008). In the wild, genetic variation and selection allows evolutionary adaptation to environmental perturbations (Frankham *et al.*, 2010; Taylor, 2003). However, genetic adaptation to captivity can occur in just several generations (Williams and Hoffman, 2009) and potentially jeopardize restoration efforts by producing an unsustainable population with a reduced ability to persist in its natural habitat on reintroduction to the wild (Ficetola *et al.*, 2011; Williams and Hoffman, 2009).

Both inbreeding and adaptation to captivity can substantially be reduced by re-diversification through the introduction of individuals from other populations (Boakes *et al.*, 2007; Frankham *et al.*, 2010; Taylor, 2003). This involves either immigration of wild-caught individuals into captivity or the transfer of captive individuals between breeding institutions (Taylor, 2003). However, most CBPs operate under limited space and resources and these management strategies are not always practical. In addition, it is highly likely that a proportion of the captive population will not genetically contribute to the gene pool as failure to breed is common among animals in captivity (Hambler and Canney, 2004). Therefore, removing a species from *in-situ* threats is only half the battle. Successful breeding of frogs in captivity is another challenge entirely.

**Anuran captive breeding**

The amphibian extinction crisis has doubled the predicted global estimate of the total number of all species that will require *ex-situ* conservation measures over the next two centuries (Frankham *et al.*, 2010). Many frog species will go extinct before any habitat rescue measures can be implemented and as a result, captive breeding is becoming increasingly more crucial in efforts to minimize the loss of global anuran diversity.
(Bishop et al., 2012). The global zoo and aquarium community have actively embraced this challenge by endeavoring to provide resources and facilities for the accommodation and captive breeding of endangered frog and toad species. Furthermore, the recent endorsement of CBPs within zoos and aquariums by the IUCN is a huge step in the right direction in recognizing ex-situ strategies as a valuable conservation alternative (Frankham et al., 2010; IUCN, 2015).

Frogs possess a number of life-history characteristics that are advantageous for captive breeding including external fertilization, a relatively high fecundity and short generation time allowing for rapid population expansion (Griffiths and Pavajeau, 2008; Kouba et al., 2009). Furthermore, their small body size and a relatively low-maintenance husbandry regime, is more cost-effective compared to larger vertebrate species management, with the additional advantage of a hard-wired behaviour requiring no prior conditioning for wild reintroductions (Balmford et al., 1996; Griffiths and Pavajeau, 2008). However, despite being particularly suitable for CBPs, amphibians often remain neglected in zoo-based conservation programs and tend to be considered uncharismatic or not attractive enough to garner public interest or inspire potential fund-raising efforts (Balmford et al., 1996). Consequently, captive breeding is not profitable and funding for research is limited; therefore, successful and useful anuran captive breeding programs will only be realized through further financial and political endorsement worldwide.

Captive breeding has improved the status of several anuran species that would undoubtedly have gone extinct without intervention. The most dramatic species rescue mission must surely be that of the mountain chicken frog (Leptodactylus fallax), listed as critically endangered since human pressure compounded by Chytridiomycosis caused an
80% decline in the one remaining population on the island of Montserrat (IUCN, 2015). An emergency operation was activated in 2009 to airlift the remaining 50 frogs off the island to captive breeding facilities in the UK and Sweden and three years later, 33 frogs were released back onto the island and their survival is being regularly monitored (Wren, 2009). Further demonstrating the value of CBPs is the Kihansi spray toad (*Nectophrynoides asperginis*). *N. asperginis* was declared extinct in the wild in 2009 due to habitat modification restricting the breeding sites of this micro-endemic species (Channing *et al.*, 2006; IUCN, 2015). Fortunately, a rescue mission was implemented in time and there are currently 6000 Kihansi spray toads residing in biosecure facilities at the Bronx and Toledo zoos successfully producing offspring to support reintroduction efforts (Amphibian Ark, 2015). Since 2013, soft-release experiments in addition to full reintroductions were initiated and will be monitored for five years (IUCN, 2015). Perhaps best epitomizing the credibility of an *ex-situ* rescue effort is the plight of Mallorcan midwide toad (*Alytes muletensis*). Following successful captive breeding of just 20 individuals and subsequent release of 76 captive-born tadpoles into Mallorca in 1989 there are now 19 sustainable populations spread over 200 square kilometers. Consequently, the threat level of *A. muletensis* has decreased from critically-endangered to vulnerable (IUCN, 2015).

**Reproductive failure in anuran captive breeding**

In the aforementioned cases, the species concerned bred naturally in captivity but this is not always the case. In fact, reproductive failure is a common problem in many captive breeding programs. Reproductive dysfunction or reduced reproductive capacity can occur in both sexes and manifest as a lack of behaviors such as amplexus and calling
in males and the absence of phonotaxis, failure to ovulate or a reduction in egg mass size and quality in females (Kouba et al., 2012a). Further examination of the neuroendocrine basis of amphibian reproduction could provide an important means of understanding how disruption of reproduction could occur under perturbed environmental conditions, resulting in reduced fertility and declining populations. (Tsai, 2011)

**Absence of reproductive cues**

The life-history of anurans is strongly influenced by the external environment including weather and climate, with temperature of particular importance (Corn, 2005). Reproductive behaviour, specifically, is characterized by a complex synergistic relationship between a suite of environmental stimuli and the neuroendocrine responses that regulate gonadal activity and breeding. (Corn 2005; Tsai, 2011). Reproductive cues vary depending on species and geography (Mann et al., 2010) and include temperature, as well as other, non-thermal, cues for reproduction include rainfall, humidity, photoperiod (sometimes) and food availability (Mann et al, 2010; Rastogi et al., 2011). Photoperiod has been shown to affect ovarian development in the female bullfrog (Rana catesbeiana), which requires a 12/12 hour light/dark cycle for optimum ovary maturity (Easley et al., 1979; Wright and Whitaker, 2001). Similarly, inappropriate temperature can also decrease gametogenesis (Wright and Whitaker, 2001). Temperature is a major influence on the reproductive patterns of high elevation seasonal breeders (Duellman and Trueb, 1994; Rastogi et al., 2011). Gonadal growth and spermatogenesis are associated with increasing temperatures, with low temperatures tending to depress gonadal activity in temperate male anuran species (Rastogi et al., 2011).
In an ideal world, captive breeding populations would be located in the native range of the target species, allowing animals to be maintained within their natural environmental parameters (Pritchard, 1995). However, this is generally not a practical option for many species and important reproductive cues are often missing, unknown or cannot feasibly be replicated in captivity (Kouba et al., 2009). Therefore a basic understanding of the life-history of a target species, including the requisite triggers for the regulation of gametogenesis and breeding, are fundamental to captive breeding efforts (Rastogi et al., 2011; Monfort, 2014; Wright and Whitaker, 2001). With an increasing number of species facing imminent extinction there is little time to investigate the mating cues of all species in need of rescue presenting an enormous challenge for CBPs that already operate under limited resources.

**Nutritional deficiency**

Nutrition is also thought to play a significant role in reproductive dysfunction in captivity. However, the nutritional requirements of captive anurans are poorly understood (Ogilvy et al., 2012; Rastogi et al., 2011). Frogs and toads store their energetic supplies in abdominal fat bodies which can comprise several classes of lipid and protein and can synthesize steroid hormones *in vivo* (Rastogi et al., 2011). Fat body mass is directly liked to reproductive success and there are indications that in both sexes fat bodies are needed to support normal gonadal activity (Rastogi et al, 2011). The nutritional status of females might affect the numbers and size of the eggs (Duellman and Trueb, 1994) and there is evidence of a direct relationship between nutrition and oocyte growth in *Xenopus laevis* (Holland and Dumont, 1975). Removal of fat bodies in females negatively impacts the reproductive system by essentially shutting down during vitellogenesis impeding the
transfer of essential lipids and proteins to pre-vitellogenic follicles (Pierantoni et al., 1983; Rastogi et al., 2011). In males, removal of fat bodies might cause degeneration of primary spermatocytes and testicular atrophy (Rastogi et al., 2005). Therefore, maintenance of reproductive condition through an appropriately nutritious diet is crucial to optimizing reproductive success of anurans captivity (Kouba et al., 2012a). Gut-loading feeder insects with appropriate vitamin and mineral supplementation can improve reproductive function in captive anurans and goes some way towards ensuring nutritious diet. Supplementation with carotenoids has been shown to have a strong positive effect on fecundity and egg quality in a number of captive frog species (Dugas et al., 2013; Ogilvy et al., 2012).

**Stress-induced inhibition of reproduction**

The reproductive system is the first thing to shut-down when an animal is under stressful conditions consequently, stress-induced reproductive failure is a common problem for many captive breeding programs (Kouba et al., 2012a). A stressor, as defined by Narayan (2013), is a ‘noxious stimulant, which exposes the amphibian to energetic costs outside of that required for predictable daily and seasonal requirements’. Stress elicits many neural and endocrine changes which can profoundly affect reproductive function (Kaiser et al., 2015). It is well established that stress increases cortisol levels in captive anurans and that this can inhibit critical endocrine pathways that regulate gametogenesis and breeding (Carr, 2011). Chronic environmental stress has been linked to a precipitous decline in sperm count and viability in White’s tree frog (*Litoria caerulea*) but the underlying mechanisms have not yet been fully elucidated (Kaiser et al., 2015).
Reproductive behaviour in anurans is energetically expensive and depletes energy stores that may be needed in the future i.e. to evade a predator (Carr, 2011). In amphibians, a stress response activates the hypothalamo-pituitary-interrenal (HPI) axis, a system of neural transmitters and hormone responses (Narayan, 2013). A stress event causes the HPI axis to activate the hypothalamic release of corticotrophin-releasing hormone which travels to the anterior pituitary where adrenocorticotropic (ACTH) hormone is released into the blood (Narayan, 2013). The ACTH released into the blood stimulates the release of glucocorticoids, specifically corticosterone in amphibians (Narayan, 2013). Elevated plasma levels of corticosterone can influence gametogenesis, sex steroid synthesis, reproductive behavior, and enhance susceptibility to pathogenic infections and parasite (Carr, 2011; Kaiser, et al., 2015). Regulating the stress response are a suite of physiological changes that have evolved to inhibit reproductive behavior when under threat (Narayan, 2013). A major problem for captive breeding is that the physiological response to stress is induced by numerous stimuli and if prolonged can chronically elevate glucocorticoids, influencing the long-term reproductive capacity of captive animals (Carr, 2011).

**Developing ARTs for Captive Breeding**

Where natural mating cannot be achieved in captivity, assisted reproductive technologies (ART) such as exogenous hormone treatment for the stimulation of gamete release, artificial fertilization (AF) for population augmentation, and gamete cryopreservation for genetic security, can be used to circumvent the dearth in information on anuran reproductive physiology. ARTs are valuable conservation tools and confer several advantages to a CBP by: (1) increasing the carrying capacity without increasing
population size (2) reducing the costs of animal maintenance through cryobanking
gametes (3) increasing genetic diversity, and (4) facilitating genetic management and
gamete security (Clulow et al., 2012). The incorporation of ARTs into amphibian
conservation is relatively recent but a number of key findings have occurred over the last
few years.

**Novel hormone therapies**

Exogenous hormone therapy can be used to manipulate various stages of the
hormone cycle by substituting for missing environmental or endogenous cues (Wright
and Whitaker, 2001). The hormonal components of the HPG axis in amphibians are
basically similar to those described for mammals (Licht and Porter, 1987; Licht and
McCreery, 1985). However, important variations in the intricacies of these interactions
must be taken into account in attempts to understand or manipulate the endocrine system
(Clulow et al., 2014). There are two widely used exogenous hormone treatments: human
chorionic gonadotropin (hCG) and synthetic analogs of gonadotropin releasing-hormone
(GnRHa). These hormones substitute for (hCG), or stimulate the production and release
of endogenous gonadotropins. A greater knowledge of the HPG axis can allow us to
better override the reproductive cycle through artificial means, thereby inducing
reproductive behaviour for a better understanding of (1) the brain factors regulating the
pituitary, especially gonadotropin-releasing hormone (GnRH) (2) the pituitary
gonadotropins and potentially other hormones that control the gonads, (3) the gonadal sex
steroids that control feedback mechanisms on the hypothalamus and pituitary (Clulow et
al., 2014).
A number of practical considerations that must be taken into account when developing novel hormone regimens for spermiation induction, such as: the type of hormone that is required; the hormone source; dosage parameters for initial investigations of the hormones, and regularity of hormone administration (Goncharov et al., 1989). Following preliminary investigations, further exploration of the hormone protocol is warranted, to ensure that the protocol is refined and optimized. This involves a comprehensive hormone efficacy study to establish a dose-dependency curve for the particular hormone tested, in order to ascertain the most effective minimum dose and ensure that a hormone or dosage is not ruled out prematurely (Kouba et al., 2012a). In addition, the gamete collection timeline should be extended and/or incorporate regular collection times to ensure that peak spermiation response to hormone treatment is not overlooked. To highlight this point: following exogenous hormone administration, the Mississippi gopher frog initiates a spermiation response within 30 min which last up to 2.5 h (Kouba et al., 2011). In contrast, peak sperm production in the boreal toad is between 3 and 7 h post-hormone administration, and continues up to 24 h PA. It is important to ensure that the spermiation timeline has been correctly established.

The most widely used hormones for the stimulation of gamete production in the anurans can be classified as ‘first generation’ and ‘second generation’ hormones (Kouba et al., 2012). First generation hormones act directly at the level of the gonads to induce the synthesis and release of testosterone for spermiation in males, and progesterone for final egg maturation, ovulation and spawning in females (Kouba et al., 2012a; Tsai, 2011). These first generation hormones are typically used for the collection of gametes for artificial fertilization (AF) and research purposes (Kouba et al., 2012b). The
mammalian glycoprotein hormone human chorionic gonadotropin (hCG) is a LH analog with direct gonadal action and is commonly used in hormone therapy for artificial fertilization due to the simplicity of dosage preparation by standardized units (International Units) and its wide availability (Clulow et al., 2014; Kouba et al., 2012a). In contrast, second generation hormones, such as the synthetic GnRH agonist (GnRHa) act at the level of the pituitary to induce the release of endogenous LH and FSH, thus promoting a more natural hormone cascade which is more likely to elicit a behavioral response (Kouba et al., 2012a; Tsai, 2011; Wright and Whitaker, 2001). Therefore, the desired outcome of a hormonally-assisted breeding event is somewhat influential over the specific hormone employed. For example for artificial fertilization and research purposes, hormone induction at the level of the gonads via hCG is suitable enough for the production and collection of gametes. However, if natural mating is preferred then a GnRHa approach might be more appropriate.

The application of gonadotropin-releasing hormone agonists

Treatment with gonadotropin-hormone releasing hormone agonist ([GnRHa] also referred to in some literature as luteinizing hormone-releasing hormone [LHRH]) is a highly effective method to induce ovulation and spermiation in a number of anuran species (Clulow, et al., 2014; Kouba et al., 2012a). A number of GnRH agonists have been developed that induce release of LH (Clulow et al., 2014). Exogenous administration of a specific GnRHa (des-Gly₁⁰, D-Ala⁶, Pro⁹-GnRH-ethylamide), a synthetically modified form of mammalian GnRH (mGnRH), is thought to be the most effective of the GnRH agonists in terms of enhanced receptor binding and endurance (Clulow et al., 2014).
With any hormone, caution should be exercised (Wright and Whitaker, 2001) as species-specificity is highly likely and current knowledge is limited to a small number of species, relatively speaking (Wright and Whitaker, 2001). Furthermore, different analogs can induce different reactions and a number of factors have been shown to affect response to GnRH administration, including: time of day the injection is administered; seasonality; and, sex (males tend to yield a greater response) (Daniels and Licht, 1990; Easley et al, 1979; Wright and Whitaker, 2001). Differences in sensitivity to GnRH on a species level, could be a result of reduced binding of the agonist to the anuran GnRH receptor (Clulow et al., 2014). Immunostaining of nerve tracts with antiserum to GnRH in the brains of various anuran species indicated a complex distribution of GnRH expressing cells, as indicated by variable staining (Hayes et al., 2013). In addition, an absence of staining in some species suggests a structural divergence in the molecule. There is evidence to suggest that administration of a DA antagonist in combination with GnRHa might optimize LH release for less receptive species (Trudeau et al., 2010, 2012; Figure 2). The use of antidopaminergic drugs in combination with GnRHa has been successful in several frog species (Trudeau et al., 2010).

GnRH agonists induce spermiation in a number of frog species, including: (Waggener and Carrol, 1998a; Goncharov et al., 1989; Obringer et al., 2000; Wright and Whitaker 2001; Rowson et al, 2001; Kouba et al, 2012b, Browne et al, 2006; Kouba et al, 2009, Mansour et al., 2010, Mann et al., 2010; Byrne and Silla, 2010; Silla., 2010, 2011; Uteshev et al., 2013). GnRHa are also used in females of a number of species to induce final egg maturation, ovulation and spawning (Waggener and Carroll, 1998, Wright and Whitaker, 2001; Roth and Obringer 2003, Michael and Jones, 2004 Kouba et
GnRH analogs act at the level of the brain (Figure 2) to induce a more natural endogenous hormone response and as such, are generally used in captive breeding where natural mating may be preferred over artificial fertilization (Kouba et al., 2012a).
Figure 2   Exogenous hormone manipulation of the HPG axis

The Hypothalamic-pituitary-Gonadal (HPG) axis featuring hormone cascade events following (1) exogenous administration of GnRHa. GnRH receptor binding at the anterior pituitary (AP) releases endogenous Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). LH and FSH act at the level of the gonads to stimulate steroidogenesis in the testes, follicular growth and ovulation in the ovaries and yolk formation (vitellogenesis) in the liver. The exogenous administration of hCG (2) mimics the bioactivity of LH at the level of the gonads. Dopamine (DA) antagonists (3) increase receptor binding in the AP to induce a stronger LH surge from the pituitary.

However, GnRHa has been shown to cause desensitization of the pituitary in mammals where the pulsatile nature of GnRH release and need for non-continuous stimulation is necessary to avoid pituitary desensitization (Gallo et al., 1980). In contrast,
studies to date indicate that amphibians do not share the same temporal requirements and prolonged continuous exposure to GnRHa may optimize response (McCreery and Licht 1983). (Clulow et al., 2014; Kouba et al., 2012a).

The application of exogenous chorionic gonadotropins

The mammalian glycoprotein hormone human chorionic gonadotropin (hCG) is an LH analog that directly stimulates the gonads to induce the synthesis and release of testosterone in males, and egg maturation, ovulation and spawning in females (Tsai, 2011; Figure 2). Due to its activity directly at the level of the gonads, administration of hCG is generally employed in captive breeding efforts for the collection of gametes for artificial fertilization, as opposed to the induction of natural breeding.

Exogenous hormone administration of hCG, either alone or in combination with a synthetic GnRH agonist (GnRHa), is routinely used in a number of anuran species to stimulate sperm release, which is expressed in urine (spermic urine) (Browne et al., 2006; MacDonough et al., 2015; McKinnell et al., 1976); Mann et al., 2010; Obringer et al., 2000; Rowson et al, 2001; Shishova et al., 2011; Uteshev, et al., 2013; Waggener and Carroll, 1998a). A number of studies indicate that hCG alone produces a greater spermiation response than in combination with GnRHa in many Bufonidae species, including Anaxyrus fowleri (Kouba et al., 2012a; Kouba and Vance, 2009; McDonough et al., 2015), Anaxyrus baxteri (Kouba et al., 2009; Kouba and Vance, 2009; Browne et al., 2006), Anaxyrus americanus (Kouba et al., 2012b), Xenopus laevis and Rana catesbeiana (Easley et al., 1979), R. pipiens and L. laevis (Waggener and Carroll, 1998a), and Peltophryne lemur [pers. observ.].
However, as with most exogenous hormone treatments there can be high
variability in response between species. This could due to a number of factors: (1)
hormone-specific β subunits in hCG may account for any species-specificity in
effectiveness at inducing spermiation and there may be genus-based incompatibility in
receptor affinity (Tsai, 2011), (2) gonadal response to gonadotropin hormones may also
develop on local mechanisms, but the role of paracrine and autocrine factors are not yet
fully understood (Pierantoni, et al., 2002) (3) overstimulation and subsequent
desensitization of hCG receptors through overuse of hCG might initiate an immune
response and the production of antibodies that render the hormone ineffectual (Swanson
et al., 1995; Kouba et al., 2012). It is important to consider all the above factors when
developing a hormone protocol for the induction of gametes.

Artificial fertilization

The use of hormone therapy to promote breeding in captive anurans is not a novel
technique, this practice has been employed for several decades, albeit in a limited number
of laboratory species, mostly for biomedical research. Indeed, the first pregnancy tests
involved the administration of exogenous hCG to male frogs for stimulation of sperm
production (Galli-Mainini, 1947). The biological advantage of model frog species is that
they are characterized by external fertilization and a relatively large egg size (around
1mm for the common model frog Xenopus laevis), making them excellent vehicles for
early vertebrate development studies, embryonic manipulation and reproductive research
(Duellman and Trueb, 1994). The relatively simplistic nature of reproduction in the
majority of anuran species means that techniques such as artificial fertilization (AF) are
reasonably straightforward to apply, on a practical level (Kouba et al., 2009; Wright and
Whitaker, 2001). Following hormone stimulation, gametes, expressed from males and females, can be mixed together in a dish for fertilization and the resulting embryos left to develop through metamorphosis with minimal input (Kouba et al., 2009). Successful AF is contingent upon the reproductive condition of the animals and simultaneous collection of gametes, which requires synchronicity of the reproductive cycle of both sexes (Wright and Whitaker, 2001). Where this cannot be achieved, exogenous hormones might be necessary to facilitate this obstacle.

The majority of early AF studies were conducted using gametes collected post-mortem, which is not a viable option for an endangered species. In 1998, Waggener and Carrol (1998a) introduced the concept of artificial fertilization using live anurans (8 species of Lepidobatrachus spp.) following exogenous administration of GnRHa. Sperm was obtained by cloacal lavage to stimulate the release of urine containing sperm from 7 of the 8 species trialed; artificial fertilizations resulted in tadpoles from *L. laevis* and *L. llanensis*. This breakthrough technique provided a means to employ AF in endangered anuran species without sacrificing the donor individuals through the collection of hormonally-induced spermic urine. This groundbreaking paper prompted further exploration of the use of this technique in endangered anuran species. The Wyoming toad was the first endangered amphibian species to be successfully bred by AF using hormonally-induced spermic urine at the Memphis Zoo in 2004 (Browne et al., 2006). Following this, a number of other species have been bred using these AF techniques including: the dusky gopher frog (*Lithobates sevosa*); the boreal toad (*Anaxyrus boreas boreas*); the Puerto Rican crested toad (*Peltophryne lemur*), and; the Corroboree frog (*Pseudophryne corroboree*) (Kouba et al., 2011; Kouba et al., 2012; Diane Barber, pers.
comm.; Byrne and Silla, 2010) and the production and provision of tadpoles for release from *A. baxteri, A. b. boreas* and *P. lemur* (Browne *et al.*, 2006; Kouba *et al.*, 2009; Kouba and Vance, 2009; Diane Barber, pers. comm.).

If a successful gamete induction protocol can be established then it is relatively simple to maximize the fertilization rates of captive frog species with the ultimate aim of reintroductions into the wild. The last decade has produced a number of studies into hormone protocol development for gamete stimulation in several endangered anuran species. However, the sheer diversity of reproductive strategies that was paramount to the evolutionary success of the anurans presents many challenges to the development of exogenous hormone protocols, which tend to be established on a species-specific basis (Clulow *et al.*, 2012). Further research into anuran reproductive endocrinology is vitally important for the future development of ARTs and the ultimate success of captive breeding programs where natural mating cannot be achieved (Clulow *et al.*, 2014).

**Gamete cryopreservation**

The term cryopreservation is used to describe the freezing and long-term storage of living cells, tissues and organs. The process of cryopreservation comprises the entire procedure from sperm preparation and dilution to post-thawing maintenance of functional capability; each stage can adversely affect the functionality of spermatozoon (Watson, 1995). Lazzaro Spallanzani (1729-1799), an Italian priest and scientist, is credited with some of the early experiments in cryobiology, keeping frog sperm viable after cooling in snow and re-warming (Elder *et al.*, 2005). However, the most important year for cryobiology wasn’t for another two centuries when Luyet and Hodapp first used sucrose to dehydrate frog sperm prior to immersion in liquid air (-192° C) and achieved 60%
motility on recovery (Luyet and Hodapp, 1938). A few years later, Polge et al. (1949) inadvertently stumbled upon the cryoprotective properties of glycerol and accelerated gamete cryopreservation research (Arav and Natan, 2013; Watson, 1995). In the last fifty years, a vast number of studies in a wide range of species have been conducted to ascertain protocols for the successful freezing of gametes (Watson, 1995). The measure of ‘success’ regarding cryopreservation is determined by the recovery of motility and functional capacity, and has been reported in many vertebrate species (Watson, 1995). Initially, gamete cryopreservation was utilized for clinical and medical research purposes until the agricultural industry picked up on the benefits of being able to maintain gametes for long-term storage. More recently, cryopreservation has been recognized as a valuable conservation tool for securing the genetic diversity of declining and rare species to support captive breeding programs.

The process of cryopreservation is a major stressor to spermatozoa, reducing both functionality and survival (Holt et al., 2003). Successful cryopreservation requires the protection of cells from damage by the prevention of ice-crystal formation. There are many variables to consider when developing a cryopreservation protocol but the two governing factors are the freezing solution used to protect the cell from damage, and the rate at which the cells are cooled.

**Cryoprotectant solution**

To preserve viable cells through cryopreservation, as much water as possible must be removed from inside the cell without compromising its integrity. The volume of water in sperm cells is ~50% (Newton et al. 1999). A commonly used method for cell dehydration is by exposure to hypertonic conditions during cryopreservation through
exposure to cryoprotectants. There are a number of cryoprotective agents (CPAs), or freezing, solutions that are routinely used for gamete freezing across a broad range of species (Watson and Holt, 2001). Cryoprotectants can be divided into two main types: permeating and non-permeating. Permeating cryoprotectants tend to be non-electrolytes of lower molecular weight with a high solubility in water, whereas non-permeating cryoprotectants have higher molecular weights (Swain and Smith, 2010).

There are a wide range of cryoprotectant solutions used to protect the cells during the freezing process. Permeating cryoprotectants can have differential toxicity depending on the type, temperature and time of exposure, which can all affect the rate of penetration (Swain and Smith, 2010). Dimethyl sulfoxide and glycerol have traditionally been demonstrated to be the most effective agents for cryopreservation, however, glycerol can also be one of the most toxic (Watson and Fuller, 2001). Other cryoprotectants include: Dimethyl formamide, dimethyl acetamide, propylene glycol, and polyethylene glycol. Two important procedures relating to permeating cryoprotectants are the addition of a cryoprotectant to a cell before freezing and the removal of the cryoprotectant after freezing and thawing, both of which can cause significant osmotic stress that might damage the cell (Gao, et al 1995).

Permeating cryoprotectants lower the freezing point of the solution to extend the time for cell dehydration and ensure that sufficient intracellular water leaves the cell before freezing (Shaw and Jones, 2003). Once ice crystallization is initiated, ice crystals will grow, drawing water out of the cell through osmotic dehydration. This increases the intracellular solute concentration, increasing the viscosity of the solution until it reaches its eutectic temperature at -40° C (Mazur et al., 1981). Non-permeating cryoprotectants
typically include monosaccharide sugars, disaccharide sugars, polysaccharides and macromolecules (Swain and Smith, 2010). Non-permeating cryoprotectants have two main functions: cell dehydration during the cooling process by increasing the osmolality of the extracellular space, prevention of osmotic swelling during the thawing procedure (Lahnsteiner et al., 1996).

**The causes of cryoinjury**

There are two major types of damage to a cell during the cryopreservation process: intracellular ice formation and solution effects. For the long-term low-temperature storage of gametes, temperatures are required to be below -100 °C (Mazur et al., 1980). At this temperature there is a risk of ice crystal formation and therefore the liquid become more concentrated and draws intracellular water out to the point where the solution becomes solid (Watson and Fuller, 2001). Water also becomes drawn out of the gametes, and as it does, the cell volume is reduced and this is one of the most crucial points in the freezing process. If the water is drawn out of the cell too quickly due to the cooling rate being too high, enough water isn’t drawn out of the cell and intracellular ice formation will occur, disrupting cellular structure and function (Watson and Fuller, 2001). An excessively slow cooling rate, however, will cause ‘solution effects’ through the long-term exposure of the cell to hypertonic conditions in the residual liquid fraction (Watson and Fuller, 2001). The exact cause of the solution effects has not been conclusively determined but is thought be due to one or other of the following: high salt concentration, physical reduction in liquid space compacting the cells, the physical relationship between the cells and surrounding ice, or pH fluctuations that can denature
proteins (Watson and Fuller, 2001). An optimum cooling rate can minimize the damaging effects of solution effects (Mazur et al., 1972)

Conventional cryopreservation utilizes a ‘slow rate’ of cooling (<40° C/min) and cryoprotectant concentrations in the range of 5-15%. This is compared to the alternative ‘ultra-rapid’ freezing method (vitrification) that utilizes cooling rates of >1000° C/min and cryoprotectant concentrations in the range of 40%-60% (Cuevas-Uribe et al., 2011). There is an inverse relationship between the rates of cooling and warming, and the concentration of cryoprotectant (i.e. the faster the cooling and warming, the lower the concentration of cryoprotectant needed and vice versa) (Mazur et al., 2008).

**Gamete cryopreservation in the anurans**

*Spermatozoa cryopreservation*

Given that two of the earliest studies in cryobiology involved the cryopreservation of frog sperm (Spallanzani (1776) and Luyet and Hodapp (1938) it is unfortunate, in the face of the amphibian extinction crisis, that gametes from only a small number of frog species have since been successfully cryopreserved. Several decades after Luyet and Hodapp’s achievement, Barton and Guttman (1972) reported the successful cryopreservation of the testes macerates of *Bufo americanus*. Several decades later, cryopreservation of testicular sperm and the successful recovery of post-thaw motility has been achieved in a number of frog and toad species, including: *Rana temporaria* (Kaurova et al., 2006; Mansour et al., 2010); *Bufo marinus* (Browne et al., 1998, 2001, 2002ab); *Lithobates sylvatica* and *L. pipiens* (Beesley et al., 1998; Costanzo et al., 1998; Mungano et al., 1998); *Litoria brevipalmata*, *L. fallax*, *L. subglandulosa*, *L. leseuri*, *L. phylochroa*, *L. peroni*, *L. nausata*, *L. latopalmata*, *L. denata* and *Limnodynastes peroni,*
Crinia signifera, Philoria sp., and Pseudophyrne bibroni (Browne et al., 2002c); Eleutherodactylus coqui (Michael and Jones, 2004); and, Xenopus laevis and X. tropicalis (Bucholz et al., 2004; Mansour et al., 2009ab; Sargent and Mohun, 2005). However, to date, a very limited data set exists on the successful application of cryopreservation techniques to threatened or endangered frog and toad populations (Kouba and Vance, 2009). Until recently, a major hurdle in banking down valuable captive anurans was the nature of gamete collection, which required sacrificing the donor male to excise and macerate the testes (Kouba and Vance, 2009; Kouba et al., 2013). For species of conservation concern this is not a viable option, other than to opportunistically collect gametes from naturally deceased individuals.

The successful cryopreservation of spermatozoa collected by non-lethal methods has since been reported in three different anuran species: Rana pipiens (Hopkins and Herr, 2007), Rana temporaria (Shishova et al., 2011), and Pelophylax lessonae (Uteshev et al., 2013). This technique involves exogenous hormone administration to stimulate spermiation, and the recovery of spermatozoa in urine (spermic urine), either by cloacal lavage (Hopkins and Herr, 2007), or by abdominal massage (Shishova et al., 2011; Uteshev et al, 2013). This heralds a breakthrough technology in the evolution of amphibian biobanking for rare, declining and endangered species. In the intervening years, however, there have been few reports of successful application of this technique for the purpose of spermatozoa cryopreservation (Figure 3).
Anuran sperm cryopreservation dates back to 1938 when Luyet and Hodapp reported the first successful recovery of spermatozoa motility from a frog species (unknown). Since then, testicular spermatozoa from a number of other anuran species has been successfully cryopreserved. In 2007, Hopkins and Herr (2007) reported the first successful cryopreservation of sperm from live anurans, providing a means to preserve the genetic diversity of endangered frog and toad species.

Gamete cryopreservation often requires modification at a species level (Watson and Holt, 2001), and with a vast diversity in reproductive modes, anuran cryopreservation techniques are highly likely to follow this pattern. Indeed, the limited information on anuran spermatozoa cryopreservation so far shows that a range of different CPAs and concentrations have achieved variable results, chiefly dimethyl sulfoxide (DMSO), dimethyl formamide (DMFA), glycerol, and sucrose. Therefore, further investigation into sperm cryopreservation protocols for anuran gene banking through the use of common research models, and the application of successfully developed techniques to target species, must be pursued more vigorously.
There are several drawbacks to using spermic urine over testes macerates for cryopreservation. Firstly, the sperm concentrations obtained from testes macerates tend to be orders of magnitude above sperm concentrations of spermic urine (Kouba et al., 2013). This is problematic in the freezing process which requires sequential dilutions which reduce sperm concentration. Because anuran sperm are immotile in the male reproductive tract until being released into a hypotonic environment when the decrease in osmolality activates sperm motility as they spawn in water (Duellman and Trueb, 1994). For cryopreservation spermatozoa are held in a cryoprotective solution at osmolalities > 300mOsm [pers. observ.] compared to spermic urine which is usually between 20 – 80 mOSm [pers. observ]. Therefore, on thawing, activating the sperm for artificial fertilization, requires further dilution in water. Consequently, this method requires relatively high concentrations and volumes of spermic urine and thus, may not be an appropriate freezing technique for small anuran species that do not release large volumes of spermic urine. Spermatozoa vitrification with regards small species might benefit from exploring vitrification (ultra-fast freezing) techniques which tend to use smaller volumes of sperm sample.

The development of a universal cryopreservation protocol for the anurans will require a systematic comparative study of the reproductive physiology and endocrinology of each species to ascertain fundamental characteristics of the spermatozoa (Beesley et al., 1998). Spermatozoa are highly specialized end-differentiated cells comprising common organelles as well as those with specific reproductive purposes, such as an acrosome and proximal centriole (Wildt 2000). Furthermore, variation may exist in the membrane structure or physiology and its permeability to cryoprotectant solutions
(Beesley et al., 1998). Reproductive techniques that work for one species have little relevance to another Therefore, a knowledge of the biology, including ultrastructure of anuran gametes and the metabolism and motility in relation to cryopreservation is important for the design and development of a sperm cryopreservation technique (Hoar et al., 1983; Wildt, 2000).

**Oocyte cryopreservation**

Genetic banking in amphibians is currently focused on freezing sperm cells and, to date, a technique for oocyte or embryo cryopreservation has not been reported. This presents a problem as the preservation of a species requires both male and female gametes. While cryopreservation of sperm is a valuable, the inability to store anuran eggs or embryos the same way places major limitations on this technique as an ART. Cryopreservation of anuran eggs would be hugely beneficial to anuran conservation and the genetic management of CBPs by providing an additional source of genetic lineage through maternal lines. Anuran eggs have similar biophysical properties to fish eggs, and many decades have been spent on achieving successful cryopreservation to no avail (Liu et al., 1999, 2000). The major impediment is thought to be related to a sizeable yolk content, which is thought to have poor permeability with regards cryoprotectant and water, resulting in intracellular ice formation due to inadequate dehydration of the cell during cryopreservation (Clulow et al., 2014) anuran eggs is thought to be a result of the sizeable yolk content is likely to be an issue in the cryopreservation process. In the face of these challenges a successful outcome for preservation of the female germ line does not look encouraging. While cryopreserved sperm from a gene bank of frozen captive or wild populations is extremely valuable to genetic management, without cryopreserved
eggs from the same stock, live frogs can only be produced from the stored samples by androgenesis (Hoar et al., 1983). This would involve several generations of back-crossing, and the loss of the mitochondrial lineage and subsequent gain of host cell mtDNA (Penman, 1996). Therefore, it might be wise to take a different approach with regards cryopreservation of the maternal line in the anurans. Preliminary research into alternative methods of securing female genetics, such as through cryopreservation of somatic cells and isolated follicles have delivered some promising initial results, and the application of nuclear transfer technology to cryopreserved embryonic stem cells is also being investigated (Clulow et al., 2014).

**Genome Resource Banking**

Without immediate conservation measures, many species will be lost forever and it will take many millions of years to replenish the biodiversity lost through anthropogenic extinctions (Kirchner and Weil, 2000). In recent years, genome resource banks (GRBs) have been promoted as a means to preserve the extant genetics of endangered species worldwide through the cryopreservation of gametes and tissue samples for long-term storage (Ryder, 2000). In 2005, the IUCN Global Amphibian Summit listed biobanking as one of its top eleven action steps to counteract the worldwide amphibian extinction crisis (Gascon et al., 2007) and several research institutions have established genome resource banks (GRBs) within the last decade (Kouba et al., 2013). However, the current amphibian conservation crisis requires more attempts at storing germplasm to counter the threat of extinction for a growing number of vulnerable species in the wild.
GRBs offer enormous potential for the breeding management of captive populations and there is growing recognition of the need to increase efforts in the collection and preservation of frozen DNA, cells and tissue samples from endangered species (Ryder, 2000). The development of genetic resource banks (GRBs), which contain gametes as well as non-reproductive tissues, afford many advantages to a captive breeding program (CBP), including: 1) safeguarding genetic diversity against loss through disaster or disease; 2) securing valuable founder population genetics for future breeding, generations after the death of the donor individual; 3) facilitating the transport of genetics between breeding institutions without compromising animal welfare or biosecurity; 4) providing a means to increase the effective population size without housing additional animals; 5) facilitating gamete synchronization for artificial fertilization through the year-round provision of gametes; and, 6) providing a potential resource for securing genetics from wild populations to diversify CBPs (Frankham et al., 2010; Kouba and Vance, 2009; Kouba et al., 2013; Wildt, 2000).

GRBs may offer the only hope of survival for many species on the brink of extinction (Ryder, 2000). Efforts to conserve biodiversity through the preservation of gametes must be stepped up by securing extant genetic diversity now before it is too late (Ryder, 2000). Immediate target species should be those most vulnerable such as critically endangered species with no captive assurance colonies in place (Kouba et al., 2013). For captive breeding purposes a population size that retains 90% of genetic diversity for 100 years is recommended (Frankham et al., 2010). Meeting this goal through natural breeding involves maintaining large numbers of breeding individuals (Holt et al., 2001). However, most CBPs already operate under limited space and resources and these
management strategies are not always practical. GRBs allow for an increase in effective population size by providing a means to increase the generation interval and effective population size, thereby reducing the risk of genetic adaptation to captivity, and the population size required to be maintained in captivity (Holt et al., 2001; Williams and Hoffman, 2009).

In the wild, genetic variation and selection allows evolutionary adaptation to environmental perturbations (Frankham et al., 2010 Taylor, 2003). However, genetic adaptation to captivity can occur in just several generations (Williams and Hoffman, 2009) and potentially jeopardize restoration efforts by producing an unsustainable population with a reduced ability to persist in its natural habitat on reintroduction to the wild (Ficetola et al., 2011; Williams and Hoffman, 2009). Both inbreeding and adaptation to captivity can substantially be reduced by re-diversification through the introduction of gametes from other populations (Boakes et al., 2007; Frankham et al., 2009; Taylor, 2003). Traditionally, this would involve immigration of wild-caught individuals into captive populations or the transfer of captive individuals between breeding institutions (Taylor, 2003). In addition to disease transmission and welfare issues associated with the transfer of individuals between breeding populations there are the logistical concerns of increasing captive population sizes. Furthermore, the removal of wild individuals for genetic augmentation of captive assurance colonies could detrimentally impact their free-living counterparts, particularly with regards small or rare populations (Taylor, 2003). GRBs provide a means to reintroduce genetics into a population to maintain evolutionary distinctness and life history traits (Isaac et al., 2007).
There are a number of factors to consider in order for a GRB to be effective and successful in the long-term. Clear conservation and scientific goals should be defined at the outset, in order to avoid misguided collections of gametes, through the coordinated production of a GRB action plan. (Wildt, 2000). The advice of geneticists will be required to ensure organized collections from free-living and zoo-maintained species that conserve both allelic diversity and heterozygosity (Kouba et al., 2013). There are other challenges to the successful establishment of a global GRB such as propriety and biopolitical issues involving the acquisition of samples from other countries (Holt et al., 2014; Wildt, 2000). Biobanking could be challenging from countries where national governments do not want to contribute materials (Wildt 2000). Therefore, the value of GRBs and their potential to enhance amphibian conservation efforts must be promoted in order generate financial support and secure resources. There are concerns among field and conservation biologists that funding GRBs is a misappropriation of resources that should otherwise be used for habitat preservation. However, GRBs do not compete for resources with other ex-situ and in-situ conservation measures and should instead be thought of as supplementary to other conservation efforts (Kouba and Vance, 2009; Frankham et al., 2010). The ultimate success of a GRB will depend a defined framework of cooperative partnerships between zoos and research facilities, robust research, and a fully integrative approach (Holt et al., 2003; Wildt, 2000).

The Use of Model Species for Research

Anurans conform to the standard criteria of the ideal research model organism in that they are inexpensive to house, easy to maintain and have a close genomic relationship with humans (Burggren and Warburton, 2007; Hellsten, et al., 2010).
Anurans have significantly contributed to many biomedical science discoveries over the last two centuries by advancing our greater understanding of fundamental biological processes and providing us a chance to better understand the human body (Duellman and Trueb, 1994). The South African clawed frog (*Xenopus laevis*) has become a ubiquitous presence in research laboratories since its introduction as a model organism at the beginning of the 20th century (Beck *et al.*, 2001; Orlans *et al.*, 1998). Frogs remain a popular research organism and several other anuran species have been recruited as scientific models, including: *Xenopus tropicalis, Rana pipiens* and *Rana castebeiana*, and some *Bufo* and *Hyla* species (O’ Rourke, 2007).

Anurans offer important contributions to our wider understanding of biological processes, conferring several key biological advantages, including: a well understood basic physiology; a taxonomic diversity well suited to comparative studies; and, a tolerance to temperature and oxygen variation (Burggren and Warburton, 2007). Furthermore, anuran species are a particularly appropriate vehicle for developmental and embryological studies in that they are characterized by external fertilization and large quantities of eggs of a relatively large size (around 1mm for the common model frog *Xenopus laevis*) (Duellman and Trueb, 1994; Hellsten *et al.*, 2010). Frogs were fundamental to clarifying the mechanisms behind fertilization as a result of the renowned ‘frogs in trousers’ experiment by Lazzaro Spallanzani in the 18th century (Birkhead *et al.*, 2009). The Italian Catholic priest and biologist used an unknown species of ‘aquatic green frog’ to confirm that fertilization requires the fusion of both gametes, thereby invalidating the previously held theory that either the egg or the sperm could separately
develop into a new life form, and undoubtedly accelerating further reproductive research as a result (Birkhead et al., 2009).

The ease and reliability with which gamete production can be stimulated in many model anuran species using exogenous hormone treatments makes them an ideal choice for reproductive studies. Indeed, exogenous hormone treatments led to the development of the first pregnancy test in the early 20th century. Urine from pregnant women injected into male frogs induced sperm production, due to the presence of the hormone human chorionic gonadotropin (hCG), secreted by cells in the placenta following implantation of the embryo (Galli-Mainini, 1947; O’Rourke, 2007).

In recent years, model frog species are increasingly being utilized for their own advantage as the current focus on amphibian conservation is encouraging the use of a host of different common frog species as research models for their more endangered counterparts. It is not appropriate to use an endangered species as a ‘guinea pig’ for hormone treatments as it is unknown what the outcome may be. Therefore, hormone protocols to initiate breeding in captivity can be verified and refined using common model species of related Genus in order to validate ‘safe’ hormone levels before administering to their imperiled relatives.

Model species: Fowler’s toad (Anaxyrus fowleri)

The Fowler’s toad (Anaxyrus fowleri) is a member of the family Bufonidae, meaning true toad, and is native to North America and listed a species of least concern by the IUCN (Hammerson, 2004). Fowler’s toad populations are generally presumed to be stable with substantial fluctuations and, despite some areas reporting local declines, the overall population is considered to be large (Hammerson, 2004). The Fowler’s toad
occupies a broad range of habitats and is widely distributed throughout the eastern United States along areas of the Atlantic coastal plain and Florida peninsula ranging from New Hampshire to southern North Carolina (Wright and Wright 1949; Conant and Collins, 1998). Their Northern range extends to Ohio’s Lake Erie and areas of southern Ontario and their southeastern distribution ranges through eastern Texas (Green, 2005).

*A. fowleri* is a seasonal, aquatic breeder, reproducing in the warmer months between April and June exploiting both temporary and permanent open shallow water bodies (Green, 2005). During the breeding season they migrate to a suitable pond to attract females with their mating call. Successful mating will result in amplexus; a mating behaviour whereby male embraces the female from behind using special pigmented thumb spines called nuptial pads (a secondary sexual characteristic) to maintain their grip throughout the reproductive event (Duellman and Trueb, 1994). Female toads lay long twin strands of eggs (approximately 3-4 mm in diameter) (Wright and Wright, 1949) and the males release urine containing sperm (spermic urine) in synchrony. A typical Fowler’s toad egg mass in the wild contains between 2,000 -10,000 eggs which generally hatch about 7 days following fertilization (Smith, 1961), depending on water temperature, and a larval phase of between 40-60 days (Wright and Wright, 1949). Both male and female Fowler’s toads usually reach sexual maturity around 2 years of age in the wild (Green, 2005) and have an average lifespan of 5 years. Due to its listing as a species of least concern, the Fowler’s toad has been used as a model species for other, more threatened, Bufonidae species.
Target species: Boreal toad (Anaxyrus boreas boreas)

The boreal toad (Anaxyrus boreas boreas) also belongs to the Bufonidae family and is one of two sub-species of the more widespread Western toad (Anaxyrus boreas) (Goebel, 2005). Historically widespread and locally common species such as the boreal toad are declining or disappearing from substantial parts of their range in the northwest United States (Corn, 2011). The geographically isolated southern Rocky Mountain population (SRMP) has experienced significant declines over the past thirty years as a result of increasing environmental pressure and widespread prevalence of the pathogenic chytrid fungus (Corn, 2011; Hammerson, 1999; Loeffler, 2001; Muths, et al. 2003). The SRMP inhabits the montane regions of Colorado, southeast Wyoming and is now thought to be extirpated from its former range in New Mexico (Loeffler, 2001). The population is currently listed as endangered in the states of Colorado and New Mexico and protected in Wyoming (BTRT, 2001). In 1995, the U.S Fish and Wildlife Service (USFWS) classified the SRMP as a candidate species for federal listing under the Endangered Species Act (ESA), however this listing was removed a decade later following concerns over the genetic distinctness of the population (Loeffler, 2001). Subsequent genetic analysis has confirmed the SRMP as evolutionarily significant and a petition for federal listing has once again been filed (Greenwald et. al, 2011). The International Union for the Conservation of Nature (IUCN, 2015) currently lists A. b. boreas as near threatened and it remains a candidate species for federal listing.

The boreal toad is a seasonal, aquatic breeder and enters a hibernation-like state for approximately eight months of the year, emerging at time of snowmelt to breed (Hammerson, 2004) in permanent ponds. Unlike the Fowler’s toad, the male boreal toad
does not exhibit a mating call, although they will chirp in breeding aggregates, and it remains unknown how the males attract the females [pers. observ.]. Once in amplexus the female releases a long string of eggs which is fertilized over several hours, and sometimes days as the male releases spermic urine. It is thought that female boreal toads may skip one or several breeding seasons while males are presumed to be annual breeders (Carey, 1993). Females and males reach sexual maturity at 6 and 4 years old, respectively. Average lifespan of boreal toads is thought be around 12 to 15 years old but have been known to live up to 20 years in captivity [Ted Smith, pers. Comm.].

In response to continuing declines in the SRMP, the Colorado Division of Wildlife (CDOW) in 1995 established a substantial captive assurance colony at the Native Aquatic Species Restoration Facility (NASRF) in Alamosa, CO, USA (Berwyn, 2012; Loeffler, 2001). The NASRF facility currently houses over 600 captive broodstock and is instrumental in restoration efforts of native wild populations through research, breeding, reintroductions and translocations [T. Smith, pers. comm.]. Since 2000, NASRF has made significant contributions to wild reintroductions and stocking various zoos and research institutions across the U.S with approximately 117,500 tadpoles and toadlets [T. Smith, pers. comm.].

A dedicated research population is used for investigations into hormone stimulation of gamete production and sperm cryopreservation to enhance the breeding operation at NASRF and augment wild reintroductions. Inconsistent natural breeding over the last decade has required the incorporation of hormone therapy into the NASRF breeding operation [NASRF staff, pers. comm.]. Each breeding season, upon emergence from artificial hibernation, hormonally-assisted natural mating (either one or both sexes
receiving hormones) has been attempted to varying degrees of success [NASRF staff, pers. comm.]. However, reproductive output remains low, with annual survival rates to tadpole ≤5% (Calatayud et al., 2015). Therefore, the NASRF breeding operation would benefit from further investigation into hormone treatments for protocol refinement.

Target species: Puerto Rican crested toad (Peltophryne lemur)

The Puerto Rican crested toad (PCR toad; Peltophryne lemur) is also a Bufonidae species and the only native toad species on the island of Puerto Rico where two separate populations exist (Barber, 2007). The PCR toad is historically endemic to the islands of Puerto Rico and Virgin Gorda, however it is now considered extirpated from Virgin Gordo after failure to observe the species in over three decades (Johnson, 1999; USFWS, 1992). PCR toads inhabit two separate regions in northern and southern Puerto Rico, however, no toads have been seen in the north for almost thirty years and are widely considered to be extirpated from that region (Barber, 2007; Beauclerc et al., 2010; Johnson, 1999). Today, the PCR toad can be found south of the island in a small pond within the Guánica National Forest and at two other adjacent locations (Angulo 2010; Beauclerc et al., 2010) and is represented by a single wild population (Barber, 2007). The greatest threat to the PCR toad are primarily human-related and include habitat loss due to urban encroachment, spraying of pesticides, the impacts of the invasive giant toad (Anaxyrus marinus) to the island and drainage of breeding ponds (Beauclerc et al., 2010; Johnson, 1999; Barber, 2007; Paine et al., 1989). The population has declined by 80% over the last decade (Angulo, 2010) to about 250 mature adults, accelerating their IUCN listing from vulnerable in 1994 to critically endangered by 2004 (Groombridge, 2010; IUCN, 2015).
Reproduction in the PCR toad is sporadic and occurs in temporary or permanent ponds, coinciding with infrequent heavy rainfall (Johnson, 1999; USFWS, 1992; Rivero et al., 1998) when males aggregate in ephemeral ponds and initiate calling behaviour to attract the female. During amplexus the female lays long strands of eggs among vegetation. It has been reported that a female can lay up to 15,000 eggs and they lay a single clutch annually (Rivero, et al., 1998). PCR toad eggs hatch in about 24 hours and tadpoles require between 18 and 25 days to complete metamorphosis (Johnson, 1999).

The Puerto Rican Crested Toad Species Survival Plan ® (SSP) was the first amphibian SSP created through the AZA in 1984 (Barber 2007, Paine et al., 1989) and has been active for more than twenty years (Barber, 2007; Johnson, 1999). The aims of the recovery program are extensive and include: increasing awareness of the predicament of Puerto Rican crested toad throughout the island using educational outreach; protection of existing habitat and construction of new breeding grounds; establishment of at least six self-sustaining wild populations; and, instigation of research projects (Barber 2007; Beauclerc et al., 2010). Two founder captive breeding colonies were established in the 1980s representing the northern population (4 individuals) and southern population (32 individuals) (Beauclerc et al., 2010). There are currently 20 zoos and aquariums throughout the US and Canada participating in the SSP for the Puerto Rican crested toad providing an ‘insurance policy’ against a disastrous event at the Guánica breeding site and a means to produce tadpoles for release (Johnson, 1999). The first reintroductions occurred between 1982 and 1992 with the release of over 3400 tadpoles and have continued every year since (Barber, 2007). To date over 110,000 tadpoles and toadlets from captive breeding programs have been released into man-made ponds in the Guánica
National Forest (Barber, 2007). Given the precarious state in which the single remaining population exists, the addition of genome resource banking to secure important gametes would be a valuable resource for the *ex-situ* conservation efforts of the PCR toad.

**Target species: Mississippi gopher frog (Lithobates sevosa)**  
The critically endangered Mississippi (MS) gopher frog (*Lithobates sevosa*) belongs to the Ranidae or True frog family and is classified as a distinct population segment of the dusky gopher frog (*Lithobates capito*) (IUCN, 2015). The MS gopher frog is now perhaps the most endangered amphibian in the United States (Thurgate, 2007). Over the past 50 years, *L. c. sevosa* has been extirpated from much of its historical range within the longleaf pine ecosystem of nine counties along the lower coast of Louisiana and Alabama, USA (Rorabaugh, 2005). Habitat destruction and fragmentation has reduced the longleaf pine by 98 percent and consequently decreased the Mississippi gopher frog population to an estimated 125 to 150 breeding adults restricted to two adjacent ephemeral ponds in the DeSoto National Forest, MS, USA. [MS Gopher Frog Recovery Team Meeting, 2013]. Ongoing threats to the habitat include habitat destruction and fragmentation, fire suppression, hydrological alterations due to residential development on nearby land, natural rainfall fluctuations causing drought and floods and contamination by toxic and sedimentation run-off (US Fish and Wildlife Service, 2015).

Adult *L. sevosa* inhabit active and abandoned burrows of gopher tortoise or other mammals in forests with open canopy and abundant ground cover and are dependent on periodic prescribed burns to clear the forest floor. Mississippi gopher frog breeding sites are isolated temporal ponds that completely dry out at certain times of the year and fire is the only known management tool that will maintain the remaining breeding pond as
suitable habitat. The gopher frog is an explosive breeder, relying on heavy rainfall to stimulate breeding events in ephemeral ponds (Rorabaugh, 2005). The reproduction of the MS gopher frog is dependent on the hydrological period of their mating ponds as they rely on gopher winter rain showers to fill the ponds to a sufficient level for tadpoles to complete metamorphosis ponds (Rorabaugh, 2005; Thurgate, 2007). Gopher frogs hardly ever skip a breeding season when pond is full enough. The lifespan of a gopher frog in captivity is around 7 years for males and 9 years for females.

Environmental variability, coupled with genetic isolation means that the population may be unable to survive an extreme event, and as such, are vulnerable to extinction. Recovery actions for the MS gopher frog include, a collaboration between the US forest service and USFWS to protect the last remaining Mississippi gopher frog population by providing more habitat for breeding. Reintroduction efforts of egg masses in rehabilitated nearby ponds has had variable success to date (USFWS, 2015).

Between 2007 and 2010, wild-caught adult gopher frogs were removed from the wild to establish captive breeding programmes in four North American zoos. Founders are bred each year and five offspring per breeding pair are retained for future breeding [MS Gopher Frog Recovery Team Meeting, 2013]. However, the Mississippi gopher frog, like many amphibian species, is difficult to breed naturally in captivity but the successful application of ARTs including IVF has increased the captive population to over 600 individuals housed at 14 zoos across North America. This not only provides important breeding populations for reintroduction programs but also affords a means to create populations for further research, such as gamete cryopreservation and ART protocol development and refinement. The Mississippi gopher frog is an ideal target
species for assisted reproductive technologies and genome resource banking for population augmentation for eventual reintroduction and genome resource banking for genetic security.
References


Hillman, S.S., Withers, P.C., Drewes, R.C., Hillyard, S.D. Ecological and Environmental Physiology of Amphibians


Moore, R. In search of Lost Frogs: The quest to find the world’s rarest amphibians. (pp. Bloomsbury Publishing Plc: London, UK. 2014.


Rowson, A. D., Obringer, A. R. and Roth, T. L. (2001) Non-invasive treatments of luteinizing hormone-releasing hormone for inducing spermiation in American (Bufo americanus) and Gulf Coast (Bufo valliceps) toads. Zoo Biol. 20(2): 63-74


Silla, A. (2011) Effect of priming injections of luteinizing hormone-releasing hormone on spermiation and ovulation in Gunther’s Toadlet, Pseudophryne guentheri. Reproductive Biology and Endocrinology. 9:68


Tudge, C. Last animals at the Zoo: how mass extinction can be stopped. Island Press, DC, USA. 1991.


CHAPTER III
HORMONALLY-INDUCED SPERM RELEASE AND SHORT TERM COLD STORAGE OF SOUTHERN ROCKY MOUNTAIN BOREAL TOAD
(ANAXYRUS BOREAS BOREAS) SPERMATOZOA

Introduction
The boreal toad (Anaxyrus boreas boreas) is a sub-species of the more widespread western toad (Anaxyrus boreas) with historical ranges along the Western Coast of North America and eastwards along the Rocky Mountains (Hammerson, 2004). The geographically isolated Southern Rocky Mountain (SRM) boreal toad has experienced significant declines over the past thirty years as a result of increasing environmental pressure and widespread prevalence of the pathogenic chytrid fungus (Corn, 2011; Hammerson, 1999; Hossack et al., 2009; Loeffler, 2001; Muths, et al. 2003). The SRM population inhabits the montane regions of Colorado, southeast Wyoming and is now thought to be extirpated from its former range in New Mexico (Loeffler, 2001). The population is currently listed as endangered in the states of Colorado and New Mexico and protected in Wyoming (BTRT, 2001). The SRM boreal toad currently has no federal protection, despite two petitions filed within the last decade to list the population as evolutionary distinct under the Endangered Species Act [ESA] (Berwyn, 2012). The most recent step towards protection was in 2012 when the US Fish and Wildlife Service (USFWS) was issued a 90-day finding by the ESA (Federal
Register, 2012). However, the required data on population status and genetic analysis failed to be presented within the 90-day limit; as such the petition was declined (Centre for Biological Diversity, 2013). The International Union for the Conservation of Nature currently lists *Anaxyrus b. boreas* as near threatened and it remains a candidate species for federal listing (IUCN, 2015).

In 1995, Colorado Parks and Wildlife responded to continuing declines of the SRM boreal toad by establishing a substantial captive assurance colony at the Native Aquatic Species Restoration Facility (NASRF) in Alamosa, CO (Berwyn, 2012; Loeffler, 2001). The NASRF facility currently houses over 600 captive broodstock and is instrumental in restoration efforts of native wild populations through research, breeding, reintroductions and translocations [T. Smith, pers. comm.]. Since 2000, NASRF has reintroduced to the wild and supplied various zoos and research institutions across the U.S with approximately 117,500 tadpoles and toadlets [T. Smith. pers. comm.]. However, despite numerous successful releases into the wild over the last decade, the internal breeding operation at NASRF is inconsistent and reproductive output remains relatively low, with annual survival rates to tadpole ≤5% (Calatayud *et al*., 2015). A major contributing factor to the low productivity of breeding efforts at NASRF, and many other captive breeding programs, is infrequent natural mating and asynchronous gamete release (Kouba *et al*., 2012b).

Reproductive physiology in anurans is characterized by complex neuroendocrine responses to a wide range of environmental and endogenous factors (Rastogi *et al*., 2011; Wright and Whitaker, 2001). In captivity important reproductive cues are often missing, unknown or cannot feasibly be replicated (Kouba *et al*., 2009). Elucidating the
underlying endocrine patterns is essential to the development of successful assisted reproductive technology and the overall success of anuran captive breeding programs where natural breeding cannot be achieved (Clulow et al., 2014). Therefore a basic understanding of the life-history of a target species, including the requisite triggers for the regulation of gametogenesis and breeding, would be advantageous (Rastogi et al., 2011; Wright and Whitaker, 2001). However, the myriad reproductive modes exhibited by the anurans and increasing number of species facing imminent extinction presents an enormous challenge for captive breeding programs (CBPs) that already operate under limited resources.

Exogenous hormones can be used to substitute for missing environmental, nutritional or social cues to induce and regulate gamete release and mating behaviour in lieu of natural breeding (Kouba et al., 2009). Hormone therapy for reproduction in anurans is not a novel technique, indeed this practice has been employed for several decades, albeit in a limited number of laboratory species for biomedical research (Wright and Whitaker, 2001). The application of hormone therapy, along with other ARTs such as artificial fertilization (AF), for the enhancement of endangered anuran CBPs is a relatively recent practice. However, progress is impeded by the limited available literature on anuran endocrine function and as such hormone regimens tend to be based on generalizations and modified on a species-by-species basis (Clulow et al., 2014; Kouba et al., 2009). The potential for species-specificity in hormone receptivity further hinders protocol development since a number of other factors must be taken into consideration including specific hormone type, dosage, response time, and frequency and timing of injection (Goncharov et al., 1989; Kouba et al., 2012b; McDonough et al.,
Consequently, establishing a successful hormone protocol is often a rate limiting step in the development of ARTs, which can be a lengthy process of trial and error (Clulow et al., 2014; Iimori et al., 2005).

The neuroendocrine basis of the amphibian reproductive system is highly comparable to that described for mammals (Peter, 1983; Licht and Porter, 1987), whereby the hypothalamic decapetide gonadotropin-releasing hormone stimulates the pituitary release of the two gonadotropin hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that regulate gonadal function (Licht and Porter, 1987; Zerani et al., 1991). LH and FSH stimulate gonadal growth, and gamete maturation and release (Rastogi et al., 2011). Two distinct gonadotropins that structurally and functionally resemble mammalian FSH and LH have been identified in several anuran species (Licht and Porter, 1987) and it is well established that pituitary release of LH is responsible for the downstream effects of ovulation and sperm release (spermiation) in the anura (Licht and Porter, 1987).

The most widely used hormones for the stimulation of gamete production in the anurans can be classified as ‘first generation’ and ‘second generation’ hormones (Kouba et al., 2012). First generation hormones act directly at the level of the gonads to induce the synthesis and release of testosterone for spermiation in males, and progesterone for final egg maturation, ovulation and spawning in females (Kouba et al., 2012a; Tsai, 2011). These first generation hormones are typically used for the collection of gametes for artificial fertilization (AF) and research purposes (Kouba et al., 2012b). For example, the mammalian glycoprotein hormone human chorionic gonadotropin (hCG) is an LH analog with direct gonadal action and is commonly used in hormone therapy for artificial
fertilization due to the simplicity of dosage preparation by standardized units (International Units; IU) and its wide availability (Clulow et al., 2014; Kouba et al., 2012a). In contrast, second generation hormones, such as the synthetic GnRH agonist (GnRHa) act at the level of the pituitary to induce the release of endogenous LH and FSH, thus promoting a more natural hormone cascade which is more likely to elicit a behavioral response (Kouba et al., 2012a; Tsai, 2011; Wright and Whitaker, 2001). Therefore, the desired outcome of a hormonally-assisted breeding event is somewhat influenced by the specific hormone employed. For instance, when AF is utilized for research purposes, hormone induction at the level of the gonads via hCG is suitable enough for the production and collection of gametes. However, if natural mating is preferred then a GnRHa approach might be more appropriate. At NASRF, a combined hormone dose of hCG and GnRHa has been administered to both male and females to initiate natural breeding, yet fertilization rates are poor [NASRF staff. pers. comm.]

A number of studies indicate that hCG at varying concentrations is particularly effective at inducing sperm release in a number of Bufonidae species, including the Fowler’s toad (*Anaxyrus fowleri*), the Wyoming toad (*A. baxteri*), the American toad (*A. americanus*) (Kouba et al., 2012b; Kouba and Vance, 2009; McDonough et al., 2015), Kouba et al., 2009; Browne et al., 2006) and the Puerto Rican crested toad (*Peltophryne lemur*) [pers. observ.], and also in a number of frog species including the African clawed frog (*Xenopus laevis*) and the American bullfrog (*Rana catesbeiana*) (Easley et al., 1979), the Northern leopard frog (*R. pipiens*) and the Budgett’s frog (*Lepidobatrachus laevis*) (Waggener and Carroll, 1998a). Kouba and Vance (2009) successfully induced a 100% spermiation response in the boreal toad, which lasted several hours, using a single
dose of 300 International Units (IU) hCG. However, hormone dose calculations in this study were based on the average body weight (BW) per treatment group and did not take into account individual BW, which can be highly variable. Thus, administration of a single hormone dose may not be an accurate method for characterizing hormone efficacy (Kouba et al., 2012a) and protocol refinement by administration of hormone dosages on a per gram basis may be more effective at inducing an optimal spermiation response.

Assisted reproductive technologies have the potential to enhance CBPs by improving gamete synchronization. Hormonally-assisted natural mating (where one or both sexes receive hormone treatment prior to mate pairing) has been attempted at NASRF over the last several years inducing breeding behavior and spawning through a combined hormone dose of hCG and GnRHα [NASRF staff, pers. comm.]. However, it is not clear whether the low fertilization rates reported are a result of ineffective hormone treatment or asynchronous gamete deposition (where only one sex releases gametes). The development of spermiation dose-response relationships for individual species, such as the boreal toad, would be advantageous to the NASRF breeding operation as well as other CBPs.

Recently, the development of a reliable and effective exogenous hormone regimen was shown to induce egg deposition in 77% of hormone-treated female boreal toads (Calatayud et al., 2015). However, spermiation response to exogenous hormone treatment has not been fully characterized and a hormone efficacy trial has not yet been described for male boreal toads. The capacity to collect and reliably predict the timing of spermiation for gamete collection would enhance the boreal toad CBP in a number of ways, including: 1) the simultaneous collection of gametes from both sexes to maximize
AF success; 2) the collection of sperm from a number of different males allowing multiple paternities within a single egg mass to increase genetic variability; 3) facilitate the synchronization of gametes for hormone-assisted natural mating; and, 4) extend sperm longevity through short-term storage methods.

Refrigeration of spermatozoa at 4°C has been effective in maintaining motility of spermic urine samples over several days in a number of anuran species, including the Fowler’s toad, the booroolong frog (*Litoria booroolongensis*), and the Mississippi gopher frog (*Lithobates sevosa*) (Germano *et al.*, 2013; Kouba *et al.*, 2011; Silla *et al.*, 2014). This technique is extremely advantageous to a CBP in maximizing the window of opportunity for achieving artificial fertilization (Silla *et al.*, 2014). Further advantages include the facilitation of male gamete exchange between breeding institutions, (thus eliminating animal welfare and disease transmission issues associated with transporting live animals) and the potential to link *in-situ* and *ex-situ* toad populations through the collection and storage of sperm from wild-caught males. In 2011, the Memphis Zoo (Memphis, TN, USA) successfully transferred chilled *L. sevosa* sperm to Omaha’s Henry Doorly Zoo (Omaha, NE, USA) for artificial fertilization trials (Kouba *et al.*, 2011), highlighting the potential use of this sperm storage technique for a nationwide cooperative breeding operation.

The aim of this study was to determine the most effective exogenous hCG dose treatment to induce optimal quantity and quality of recovered *SRM* boreal toad sperm. Hormone and dosages were selected based on doses in the literature that did not provoke detrimental health consequences. In addition, the potential for extending sperm longevity through short-term cold storage of boreal toad spermatozoa was explored.
Specifically, the study objectives were to: 1) investigate the efficacy of three different exogenous hCG treatments in inducing spermiation; 2) characterize the dose-dependent spermiation response over a 24 h period; and 3) explore the capacity to recover motility from spermatozoa maintained in cold storage at 4°C for up to two weeks post-collection. Successful development of these techniques would facilitate gamete synchronization and maximize fertilization rates to increase the reliability of the captive boreal toad breeding operation. Overall, the projected increase in annual reproductive output would safeguard genetic diversity and enhance the reintroduction efforts of the declining SRM boreal toad.

Materials and Methods

All animal procedures were conducted following review and approval by the Mississippi State University Institutional Animal Care and Use Committee.

Animals and spermic urine collection

All adult male boreal toads originated from egg masses either collected in the Southern Rocky Mountains or reared at NASRF. Male boreal toads used in this study were from a designated research population that were not part of the captive breeding program. In October 2010, male toads (n=21; 6-12 year old) were transferred from NASRF to a designated animal laboratory at Mississippi State University. Toads were allowed an acclimation period of eight weeks in order to minimize any potential stress effects on the efficacy of the hormone treatments. Toads (n=21) were housed in ventilated polycarbonate containers (46 cm L x 56 cm W x 66 cm H) with access to water and shelter and maintained at 20–23°C under standard fluorescent lights on a 12h/12h light cycle. Single-sex groups of 4-5 individuals were housed per container and offered a
variety of food items including crickets, wax worms, and mealworms, three times per week. Crickets were gut-loaded with Repashy SuperLoad© supplement (Repashy Ventures Inc., CA, USA) and dusted with Reptivite © powder (ZooMed Laboratories, Inc., Costa Mesa, CA, USA) prior to being fed out.

For the duration of a hormone trial, male boreal toads were maintained in plastic containers (35 cm L x 20 cm W x 13 cm H) holding aged tap water (2 cm depth) to ensure continuous urine production. Prior to hormone administration, toad weight and snout-vent length (SVL) were recorded and a urine sample (To) was obtained from each toad to determine the presence (spermic) or absence (aspermic) of sperm. To collect urine, males were gently removed from their holding containers and held above a 150 mm petri dish, spreading the hind limbs apart by the thumb and index finger, until urination occurred (usually within 1 min).

**Study 1: Characterizing spermiation response to three exogenous hCG treatments**

To determine the dose dependent effects of hCG administration (delivered as g⁻¹ body weight [BW]) on sperm production and motility parameters over a 24 h period male toads (n=21) were administered an intraperitoneal (IP) injection of saline containing either: 3 IU g⁻¹ (Low treatment); 10 IU g⁻¹ (Medium treatment); or, 15 U g⁻¹ BW (High treatment) human chorionic gonadotropin (hCG; cat# C1063; Sigma-Aldrich, St. Louis, Missouri, USA) in a total volume of 200 µl. Males were randomly assigned an initial dose treatment group (Low, Medium, or High; n=7 males/group). To control for variation in individual spermiation response each group of males was cycled through each hormone treatment. This experiment was conducted between January and October 2011, with at least a two month delay between each individual group treatment.
Spermic urine was collected at 2, 3, 5, 7, 9, 12 and 24 h post-hormone administration (PA). Spermic urine samples were analyzed immediately post-collection by placing a 10 µl aliquot of sample onto a glass slide under a 400x objective on a phase-contrast microscope (Olympus CX41) and counting the first 100 sperm. Spermic urine variables measured included volume, and the percentage of spermatozoa exhibiting: Total Motility ([TM]; = flagellum movement); Forward Progressive Motility ([FPM] = forward progressive movement as a proportion of total count); and the Quality of Forward Progressive Motility ([FPM-Q] = a subjective scale from 0 (no movement) to 5 (very rapid forward movement). Sperm concentration was measured by inactivating motility in a 1:10 dilution of PBS and counting on a Neubaeur haemocytometer to obtain an average sperm concentration (x 10^6 mL^-1). If spermatozoa was present in a given urine sample at a given time-point, the individual was classified as a ‘responder’. Individuals not producing spermatozoa at a given time-point were classified as ‘non-responders’ at the corresponding time-point and were not included in any subsequent analysis. Additionally, TM, FPM and FPM-Q were only included in statistical analysis if urine samples exhibited sperm concentrations ≥ 0.1 x 10^6 mL^-1 to ensure adequate concentrations for quantifying sperm motility parameters. A control group of males (n=10) was injected with a 200 µl IP injection of sterile saline to ensure there was no effect of the carrier sterile saline injection on sperm production. No toads given sterile saline only produced sperm.

**Study 2: Short-term cold storage of hormonally-induced spermic urine**

To investigate the effect of short-term cold storage on boreal toad sperm motility, a spermic urine sample that met the following sperm quality criteria: ≥ 75% TM; ≥25%
FPM; and, FPM-Q ≥ 2 was reserved from several males (n=13) and used for Study 2. Sample were maintained in a refrigerator at 4° C for a duration of 14 days and analyzed daily for TM, FPM and FPM-Q by removal of a 10 µl aliquot and evaluating as described above for Study 1. Prior to the removal and analysis, spermic urine was gently mixed to ensure a representative sample was evaluated each day.

**Statistical analysis**

Assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilks and Levene’s tests, respectively. The total number of males spermiating in response to hCG administration was compared between hCG dose treatments (Low [3 IU g⁻¹], Medium [10 IU g⁻¹] and High [15 g⁻¹]), and between each dose treatment and the control using two-tailed Fisher’s exact tests. Sperm parameters (Concentration, TM, FPM and FPM-Q collected during Study 1 were compared between each hCG treatment (Low, Medium and High) by a two-way repeated measures analysis of variance (ANOVA), using a General Linear Model (GLM) procedure. A split-plot model was used to analyze the effect of different hCG treatments (Low, Medium and High) across time (0, 2, 3, 5, 7, 9, 12 and 24 h PA) where time was the main plot factor and hCG treatment the sub-plot factor. Within the model, hCG treatment and time were fixed factors, and sperm parameters (Concentration, TM, FPM and FPM-Q) were dependent factors. Individual toad was nested within treatment to remove variation among individual motility from the error term. Significant main effects were explored using Tukey-Kramer Honestly Significant Difference (HSD) *post-hoc* tests. Male toads producing sperm (‘responders’) were included only in comparisons of responders between each treatment group and were not included in further treatment comparisons of concentration and motility parameters.
One male (ID: BM10) was determined an outlier in sperm concentration and was removed but was included in male response and sperm motility parameter comparisons. Percentage data were arcsine transformed using the transformation $\sin^{-1}(\sqrt{x})$ before further analysis.

In Study 2, a Welch’s ANOVA was used due to unequal variances, to determine the effects of duration in cold storage (0-14 Days) on sperm motility parameters (TM and FPM). The significant effect of the continuous factor of Day was explored using Tukey-Kramer Honestly Significant Difference (HSD) post-hoc tests where TM and FPM were dependent variables and Day the independent variable. Linear regression analysis was used to establish whether any correlation existed between sperm motility parameters (TM and FPM) and duration in cold storage (0-14 Days). Pearson’s correlation coefficient was applied to determine the significance of a correlation. All values are expressed as mean ± SEM and significance was established at $P \leq 0.05$. All statistical analysis was performed in SAS Version 9.4 (Cary, North Carolina, USA).

**Results**

**Study 1: Characterizing spermiation response to three exogenous hCG treatments**

The weight and SVL of male boreal toads ranged from 27.7 – 46.6 g (mean = 35.1 ± 0.9 g) and 54.9– 66.9 mm (mean = 61.6 ± 0.7 mm), respectively. Based on a per gram basis, the dose range of hCG for Low (3 IU g$^{-1}$), Medium (10 IU g$^{-1}$) or High (15 IU g$^{-1}$) treatments was 106.4 ± 2.8 IU, 367.0 ± 10.4 IU and 549.6 ± 14.9 IU, respectively.

Urine samples were successfully collected from all males at each collection time-point (2, 3, 5, 7, 9, 12 and 24 h post-hormone administration [PA]) with urine volumes
ranging from 20-3000 µL. All urine samples collected prior to hormone treatment (T₀) were aspermic.

**Male response to hormone treatment**

‘Responders’ were classified as individuals producing spermic urine at a given time-point PA, and ‘non-responders’ as males with no spermatozoa present in a urine sample at the corresponding time-point (2, 3, 5, 7, 9, 12 and 24 h PA). Within each treatment group, 24% (5/21 males), 14% (3/21 males) and 10% (2/21 males) of males administered a Low, Medium or High hCG dose treatment, respectively, were classified as non-responders across all collection time-points. The number of spermiating males within each hormone treatment group was significantly higher relative to the control (two-tailed Fisher’s exact tests; \( P < 0.05 \)). Within 2 h PA, males in all three hormone treatment groups were producing spermatozoa; however, significantly more males at this time-point were responsive to a Medium (67%) or High (68%) treatment, in contrast to 24% of males administered a Low treatment (Figure 4).

The number of responding males remained above 70% in the Medium and High treatment groups between 3–12 h PA (Figure 4). The peak male response to hormone treatment occurred at 5 h PA when 62%, 81% and 80% of males were producing spermatozoa, in the Low, Medium and High treatments, respectively (Figure 4). There was a significant treatment effect with regards to the total number of responding males (one-way ANOVA; \( F_{2,18} = 17.95; P < 0.001 \)). The Low hormone treatment produced significantly lower male responders than the Medium and High hormone treatment groups (Tukey-Kramer; \( P < 0.05 \)) at 2 h and 12 h PA (two-tailed Fisher exact tests; \( P < 0.05 \); Figure 4). There was no significant difference in response rate between the three
treatment groups at the remaining collection time-points (3, 5, and 9 h PA), with the exception of 7 h PA (Figure 4). At 24 h PA, 38%, 57% and 71% of males continued to produce spermatozoa in the Low, Medium and High treatment groups, respectively (Figure 4).

![Graph showing spermiation response of male boreal toads over 24 h following exogenous hormone administration.](image)

**Figure 4** The spermiation response of male boreal toads over 24 h following exogenous hormone administration

The number of male boreal toads ($n=21$) exhibiting spermatozoa in urine samples collected at 7 time-points (2, 3, 5, 7, 9, 12 and 24 h) post-hormone administration (PA) of a Low (3 IU g$^{-1}$), Medium (10 IU g$^{-1}$) or High (15 IU g$^{-1}$) dose of hCG. Responders were classified as individuals producing spermic urine at each time-point PA. A spermiation response was achieved within 2 h PA and peaked at 7 h PA. Between treatment totals, significantly more males responded to the Medium and High treatments ($P<0.05$), however, there was no significant difference in spermiation response between the three treatment groups (Low, Medium and High) at 3, 5, 9 and 24 h PA ($P>0.05$). Values are expressed as percentages of the total number of male responders in each treatment group within a time-point. Values superscripted with the same letter are not significantly different ($P>0.05$).
Sperm production in response to hormone treatment

There was no significant difference between all three treatment groups [Low: 3 IU g\(^{-1}\); Medium: 10 IU g\(^{-1}\); High: 15 IU g\(^{-1}\)] in sperm concentration across time indicating that all three treatments induced a similar spermiation response across the 24 h collection period ($F_{12,208} = 1.41; P=0.16$; Table 1).

<table>
<thead>
<tr>
<th>Factors</th>
<th>Concentration</th>
<th>Motility</th>
<th>FPM</th>
<th>FPM-Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>$F_{6,208} = 5.45; P&lt;0.01$</td>
<td>$F_{2,165} = 0.14; P=0.86$</td>
<td>$F_{2,159} = 1.48; P=0.23$</td>
<td>$F_{2,159} = 6.01; P&lt;0.01$</td>
</tr>
<tr>
<td>Time</td>
<td>$F_{6,208} = 7.16; P&lt;0.01$</td>
<td>$F_{6,165} = 7.00; P&lt;0.01$</td>
<td>$F_{6,159} = 11.31; P&lt;0.01$</td>
<td>$F_{6,159} = 12.76; P&lt;0.01$</td>
</tr>
<tr>
<td>Toad ID</td>
<td>$F_{48,208} = 7.20; P&lt;0.01$</td>
<td>$F_{38,165} = 4.46; P&lt;0.01$</td>
<td>$F_{38,159} = 4.82; P&lt;0.01$</td>
<td>$F_{38,159} = 4.06; P&lt;0.01$</td>
</tr>
<tr>
<td>Treatment x time</td>
<td>$F_{12,208} = 1.41; P=0.16$</td>
<td>$F_{12,165} = 0.14; P=0.91$</td>
<td>$F_{12,159} = 1.73; P=0.06$</td>
<td>$F_{12,159} = 1.70; P=0.07$</td>
</tr>
</tbody>
</table>

Statistical results for two-way analysis of variance (ANOVA) with the effects of hormone treatment over time, a nested within-treatment toad identification, and their interactions on the dependent variables of spermatozoa concentration, Total Motility, Forward Progressive Motility (FPM) and Quality of Forward Progressive Motility (FPM-Q).

However, sperm concentration of male toads significantly differed at different times following hormone administration ($F_{6,208} = 7.16; P<0.01$; Table 1). Multiple pairwise comparisons showed a significantly higher mean sperm concentration in the High treatment compared to the Low treatment at 3 h PA and 24 h PA (Tukey-Kramer; $P<0.05$; Figure 5). Sperm concentration from males in the Low treatment group peaked twice over the course of 24 h, at 2 h and 5 h PA with $0.71 \pm 0.6 \times 10^6$ mL\(^{-1}\) and $0.79 \pm 0.2 \times 10^6$ mL\(^{-1}\), respectively (Figure 5). In contrast, males administered a Medium hCG treatment produced a more sustained period of peak sperm production between 2 h - 7 h PA with higher concentrations of $1.0 \pm 0.2 \times 10^6$ mL\(^{-1}\) - $1.5 \pm 0.4 \times 10^6$ mL\(^{-1}\) (Figure 5).
Males administered a High hCG dose treatment elicited the most rapid peak spermiation response of $2.0 \pm 0.5 \times 10^6$ mL$^{-1}$ at 3 h PA, before decreasing precipitously by 5 h PA to maintain a sperm concentration of $0.9 \pm 0.2 \times 10^6$ mL$^{-1}$ – $1.1 \pm 0.3 \times 10^6$ mL$^{-1}$ between 5 – 12 h PA (Figure 5).

There was large variation in sperm concentrations between individual males within each hCG group treatment at each collection time-point. The highest within-group per time-point ranges are as follows: Low treatment at 2 h PA: $0.0025 \times 10^6$ mL$^{-1}$ to $2.8 \times 10^6$ mL$^{-1}$; Medium at 3 h PA: $0.0013 \times 10^6$ mL$^{-1}$ to $6.45 \times 10^6$ mL$^{-1}$ (3 h PA); and, High at 3 h PA: $0.005 \times 10^6$ mL$^{-1}$ – to $7.0 \times 10^6$ mL$^{-1}$ (Table 1).

![Figure 5](image_url)

Figure 5 Sperm concentration released by boreal toads over a 24 h period in response to administration of 3 IU g$^{-1}$, 10 IU g$^{-1}$ or 15 IU g$^{-1}$ hCG

Sperm concentration of urine samples collected from male boreal toads ($n=20$) at 7 time-points (2, 3, 5, 7, 9, 12 and 24 h) post-hormone administration (PA) of a Low (3 IU g$^{-1}$ [black circle]), Medium (10 IU g$^{-1}$ [white circle]) or High (15 IU g$^{-1}$ [triangle]) dose of hCG. Sperm concentration did not differ significantly between the three treatment groups across time ($P>0.05$). Values expressed are means of the concentration produced by male responders in each dose treatment group at each collection time-point ± SEM.
Spermatozoa motility parameters

Spermatozoa motility parameters, TM and FPM did not differ significantly across time between hCG treatment group (Table 1). However, the main effect of time was significant (two-way ANOVA; \( P<0.01 \); Table 1). Multiple pairwise comparisons showed that significantly more spermatozoa maintained TM between collection points 5 – 12 h and there was a significant decrease in spermatozoa TM by the 24 h collection in all three treatment groups (Tukey-Kramer; \( P<0.05 \); Figure 6.A).
Figure 6  Motility parameters of spermatozoa released by boreal toads over a 24 h period in response to administration of 3 IU g⁻¹, 10 IU g⁻¹ or 15 IU g⁻¹ hCG

Percentage of spermatozoa (A) Total Motility (TM) (B) Forward Progressive Motility (FPM) from urine samples of male boreal toads (n=20) at 7 time-points (2, 3, 5, 7, 9, 12 and 24 h) post-hormone administration (PA) of a Low (3 IU g⁻¹), Medium (10 IU g⁻¹) or High (15 IU g⁻¹) dose of hCG. TM and FPM did not significantly differ between the three dose treatments (Low, Medium and High) across time (P>0.05). Values are expressed as untransformed means of the total number of male responders exhibiting motile sperm in each dose treatment group at each collection time-point ± SEM.
Figure 6.A shows that the general pattern of spermatozoa TM remained relatively constant over the 24 h collection period. Mean spermatozoa TM ranged between 60 ± 11.1% and 80 ± 3.3%; 59 ± 4.5% and 76 ± 2.4%; and 63 ± 3.9% and 77 ± 1.7% in the Low, Medium and High treatment groups, respectively (Figure 6.A).

Spermatozoa FPM followed a similar pattern to TM and was significantly higher between the 5–12 h collection period than at 24 h (Tukey-Kramer; $P<0.05$; Figure 6.B). There was a trend towards a dose by time interaction and males in the Medium and High treatment group produced spermatozoa with a significantly higher proportion of FPM, however this was not significant (two-way ANOVA; $P=0.06$; Figure 6.B). The quality ranking of spermatozoa FPM (FPM-Q) ranged between 1 and 5 across the 24 h collection period, peaking at 7 h PA in all three treatment groups with a mean FPM-Q between a ranking of 3 and 4 (Figure 7).
Spermatozoa FPM Quality (FPM-Q) from urine samples of male boreal toads \((n=20)\) at seven different time-points (2, 3, 5, 7, 9, 12 and 24 h) post-hormone administration (PA) of a Low (3 IU g\(^{-1}\)), Medium (10 IU g\(^{-1}\)) or High (15 IU g\(^{-1}\)) dose of hCG. FPM-Q ranged between 1 and 5 across the 24 h collection period but there was no significant difference between the three treatment groups \((P>0.05)\). Values are expressed as untransformed means of the total number of male responders exhibiting motile sperm in each dose treatment group at each collection time-point ± SEM.

There was no significant difference between the three hormone treatments across time (Table 1); however, FPM-Q was significantly higher between 5-12 h than at the 2 h and 24 h collection time-point, in all three treatment groups (Tukey-Kramer; \(P<0.05\); Figure 7). Following a similar pattern to spermatozoa FPM, there was a trend towards a treatment by time interaction with FPM-Q in the Medium treatment group significantly higher than the Low treatment and High treatment \((F_{2,159}= 6.01; P<0.01)\) but this was not significant \((P=0.07; \text{Table 1})\).
The random nested factor of toad ID, representing each individual toad, had a significant effect on all sperm quality parameters indicating significant variation between individual toads ([concentration, TM, FPM and FPM-Q]; \( P < 0.01 \); Table 1).

**Study 2: Short-term cold storage of hormonally-induced spermic urine**

Daily motility analysis over the 14 day duration of cold storage at 4°C showed that overall, the number of spermic urine samples containing motile spermatozoa declined as storage time increased (Figure 8).

![Figure 8](image)

**Figure 8**  The percentage of boreal toad spermic urine samples maintaining motility over 14 days in cold storage at 4°C.

Percent spermic urine samples \((n=13)\) exhibiting spermatozoa Total Motility (TM) and Forward Progressive Motility (FPM) following cold storage at 4°C was recorded daily up to 14 days post-collection (PC). Spermatozoa TM exhibited motility in 38% of samples \((n=5)\) at Day 14 PC. Spermatozoa FPM was maintained in 15% of spermic urine samples \((n=2)\) by Day 11 PC but had ceased by Day 12 PC. The percentage of motile samples on a given day was calculated based on the initial number of samples/number of motile samples on each collection day x 100.
By Day 14, 38% (n=5 males) of spermic urine samples exhibited spermatozoa TM (Figure 8). Spermatozoa FPM was maintained in 15% (n=2) of spermic urine samples at Day 11, but by Day 12 FPM had ceased in all samples (Figure 8). Initial TM and FPM of spermic urine samples on Day 1 was 88.2 ± 1.9 % and 41.5 ± 5.1%, respectively (Figure 9).

![Figure 9](image)

The influence of time on the Total Motility and Forward Progressive Motility of boreal toad spermatozoa maintained in cold-storage for up to 14 days.

Mean percentage of spermatozoa Total Motility (TM) and Forward Progressive Motility (FPM) in boreal toad spermic urine samples (n =13) held in cold storage at 4 °C. Spermatozoa TM and FPM was analyzed daily up to 14 days post-collection (PC). Initial spermatozoa TM decreased significantly (\(P<0.05\)) from 88.2 ± 1.9 % to 27.8 % ± 4.8%. FPM declined significantly (\(P<0.05\)) from 41.5 ± 5.1% on Day 1 to 8.0 ± 0.5% by Day 11. Values are expressed as untransformed means of the total number of spermic urine samples exhibiting motile spermatozoa at each time-point ± SEM. Values superscripted by the same letter do not significantly differ (\(P>0.05\)).
There was a significant effect of day on spermatozoa TM (Welch’s $F_{13, 138} = 9.35$; $P < 0.05$) and FPM (Welch’s $F_{11, 84} = 14.97$; $P < 0.01$). Within the first 24 h of cold-storage, spermatozoa TM declined by 16% and FPM significantly declined by 24% (Tukey-Kramer; $P < 0.05$; Figure 9). By Day 3 spermatozoa TM had significantly declined to 67% motility (Tukey-Kramer; $P < 0.05$; Figure 9). Spermatozoa motility progressively declined up to Day 14 (Figure 9). FPM continued to decline precipitously to 4% at Day 10, with the exception of an increase of 50% over a 24 h period to 8.0 ± 0.5. However, Day 11 and Day 12 are represented by only 2 spermic urine samples. By Day 11 FPM had declined to 8.0 ± 0.5% motility and ceased entirely 24 h later at Day 12. In contrast, spermatozoa TM was maintained in 5 samples with 27.8 ± 4.8% at Day 14 (Figure 9).

There was a significant negative correlation ($r^2 = -0.8726$; $P < 0.05$) between mean spermatozoa TM and the number of days in cold storage (Figure 10). Spermatozoa FPM and duration in cold storage had a low negative correlation that was not significant ($r^2 = -0.4719$; $P > 0.05$) (Figure 10).
Spermic urine samples ($n=13$) from male boreal toads were maintained at 4° C for a storage period of up to 14 days. TM and FPM were analyzed daily up to 14 days post-collection (PC). Linear regression analysis was performed representing sperm motility parameters (TM and FPM vs time in cold storage [Day]). The results indicate a strong and significant negative correlation ($r^2 = -0.8726; P<0.5$) between Total Motility and Day in cold storage and a low but not significant negative correlation ($r^2 = -0.4719; P>0.5$) between FPM and Day in cold storage. Values expressed are untransformed means of the total number of spermic urine samples exhibiting motile spermatozoa at each time-point ± SEM.

**Discussion**

In the present study, the gonadotropin hormone hCG was evaluated as a potential exogenous treatment to stimulate spermiation in a temperate, seasonally-breeding anuran species. To date, a full characterization of a spermiation profile in response to hCG treatment has not been reported for boreal toads. These results provide a comparison of the efficacy of three different hCG dose treatments (Low [3 IU g$^{-1}$], Medium [10 IU g$^{-1}$] or High [15 IU g$^{-1}$]) by quantifying sperm release over time (2, 3, 5, 7, 9, 12 and 24 h).
PA). In addition, recovery of spermatozoa motility parameters (TM and FPM) from spermic urine samples maintained in cold storage was explored as a potential technique for extending sperm longevity. Hormone efficacy on spermiation response and motility recovery of short-term cold stored sperm samples are discussed separately.

**Characterizing spermiation response to three exogenous hCG dose treatments**

The results of this study indicate hCG is a viable hormone treatment for the induction and collection of sperm from male boreal toads. With respect to male response to hormone treatment, exogenous administration of a Low (3 IU g\(^{-1}\)), Medium (10 IU g\(^{-1}\)) or High (15 IU g\(^{-1}\)) hCG dose treatment successfully induced a spermiation response in sexually mature boreal toad males within 2 h post-hormone administration (PA). Males administered a Medium or High hCG dosage achieved significantly greater total numbers of spermiating males (86% and 90%, respectively). Kouba *et al.*, (2012a) achieved a 100% response rate using a single dose of 300 IU hCG in the American toad (*A. americanus*) and this dose has also been successful at inducing a spermiation response in a number of other Bufonidae including: *A. baxteri* (Kouba and Vance, 2009; Kouba *et al.*, 2009; Browne *et al.*, 2006), *A. fowleri* (Kouba and Vance, 2009; McDonough *et al.*, 2015), and previously in *A. b. boreas* (Kouba and Vance 2009). Based on body weight, the range of hCG dosages tested in this study was between an average of 367 IU and 550 IU, for the Medium and High treatment, respectively, compared to an average of 107 IU in the Low treatment. These results, in addition to the previously reported hCG trials using 300 IU suggest that between 10–15 IU g\(^{-1}\) is an effective minimum dose for inducing spermiation in the boreal toad.
There was no significant treatment by time interaction for any of the sperm quality parameters measured (concentration, TM, FPM, FPM-Q) indicating that the magnitude and duration of male response variables were not dose-dependent and were not significantly different across the 24 h collection period. Iimori et al. (2005) reported a similar dose-independent spermiation response in cane toads (*Bufo marinus*) with no significant difference between *B. marinus* injected with either 1000 or 2000 IU hCG. There were clear dose-specific differences in time and concentration of peak sperm production in boreal toads. The Medium and High treatment groups peaked earlier and for longer, between 2-7 h PA, in contrast to the Low treatment group, which peaked between 3-5 h PA. The most sustained period of high sperm productivity was between 2-7 h PA at a dose of 10 IU g$^{-1}$ hCG. This corroborates observations on cane toads (*Bufo marinus*) in which sperm concentrations were highest at maximum plasma levels of testosterone, at around 120 min PA (Iimori et al., 2005).

Sperm production exhibited a bell-shaped curve over time peaking between 5-7 h PA and dropping off by 24 h PA. This spermiation timeline is consistent with that of other Bufonidae species including the American toad, the Wyoming toad (*Anaxyrus baxteri*) and the Fowler’s toad (*Anaxyrus fowleri*) (Browne et al., 2006; Kouba and Vance, 2009; McDonough et al., 2015). The Bufonidae appear to follow the same pattern in terms of a spermiation timeline, however, it is important, when developing hormone protocols for novel species, that generalizations are not made based on the literature. Species-specificity in time to spermiation initiation and peak sperm production must be taken into consideration, even within the same Genus, to ensure that optimum spermiation response is identified.
Although the spermiation response rate was greater in both the Medium and High treatment groups (85% and 90%, respectively), it might be worthwhile exploring whether a higher dose treatment would optimize the response and approach 100% effectiveness. For example, cane toads weighing between 79- 144 g did not respond to a straight dose of 100 IU hCG (Iimori et al., 2005). However, dosages of 1000 and 2000 IU did induce a spermiation response in the cane toad, and these dosages are more in the range of a per gram hormone concentration for this species. This underlines the importance of undertaking comprehensive hormone efficacy investigations so as to ensure that a specific hormone or dosage is not dismissed as ineffectual prematurely.

There was no dose-dependency with respect to the proportion and quality of motile spermatozoa between each treatment group; however, spermic urine collected from male toads administered a Low hormone treatment exhibited consistently lower proportions and quality of spermatozoa FPM. This suggests that treatment with a sub-optimal hormone dose might induce a spermiation response with lower quality spermatozoa. The motility and quality parameters of spermatozoa did not differ significantly between the Medium and High treatment groups, indicating that sperm quality was not affected by the uppermost dose of 15 IU⁻¹ g and there may be room to explore higher dosages of hCG in *A. b. boreas*. The quality of spermatozoa decreased significantly by the 24 h collection period in all three treatment groups but sperm continued to be released by 38%, 57% and 71% of male toads in the Low, Medium and High treatment groups, respectively. Despite being of a reduced quality, if sperm collections remain a viable option over a 24 h period, gamete synchronization is more achievable as the window of opportunity for artificial fertilization is extended.
Characterization of a spermiation time-line allows CBPs to accurately gauge the optimum time for sperm collections to ensure the best quality and quantity of sperm is harvested for breeding purposes. Motility parameters (TM and FPM) were significantly higher between 5 – 12 h PA, indicating that better quality spermatozoa was induced several hours following hormone administration and is not necessarily associated with sperm concentration. Key determinants of fertilization success are the proportion of motile and viable spermatozoa so it would be recommended to continue sperm collections even after peak sperm production to obtain better quality samples, despite the lower concentration.

In contrast to other Bufonidae, sperm concentrations from *A. b. boreas* in the present study were relatively low, although they were consistent with concentrations reported by Kouba and Vance (2009). Following injection of 300 IU hCG the American toad (*Bufo americanus*) produced an average peak sperm concentration of over 12 million mL$^{-1}$. Similarly, the Wyoming toad (*Anaxyrus baxteri*) and the Fowler’s toad (*Anaxyrus fowleri*) have been shown to produce sperm concentrations in excess of $12 \times 10^6$ mL$^{-1}$ and $4 \times 10^6$ mL$^{-1}$ at peak sperm production, respectively, using hCG at similar concentrations (Browne *et al.*, 2006; McDonough *et al.*, 2015). There may be some species-specificity with regards to receptor affinity and it is possible that hCG does not provide a substantial enough LH surge to elevate the spermiation response of the boreal toad. Alternatively, the variability with regards to sperm production might indicate that the hCG protocol developed in the present study requires further refinement to establish the most effective common dose.
Within small founder populations, mating opportunities may be limited and under-representation of genetically valuable males could undermine the genetic management of the breeding program. Therefore, in order to maximize the number of males able to genetically contribute to a breeding season, it is important to ensure that a sub-optimal protocol is not assumed the most effective and additional hormones and dosages are explored. There are a number of protocol modifications that might stimulate higher sperm production and warrant exploration. Prior administration of less potent hCG ‘priming’ doses in advance of the final injection have been shown to increase the proportion of motile sperm in *Bufo bufo*, (Usteshev et al., 2011), and increase sperm yield in *Dendrobates auratus* (Lipke et al., 2009) and *Xenopus laevis* (Frazer, 1954). Mansour et al. (2010) reported that a priming dose of 3.5 IU g\(^{-1}\) doubled the size of the testes and increased testes macerate concentrations eight-fold in the European common frog (*Rana temporaria*). Moreover, it has been suggested that priming doses might condition the hypothalamic-pituitary-gonadal axis to initiate gametogenesis and final gamete maturation (Silla, 2010). If incomplete gamete maturation is responsible for a sub-optimal spermiation response, the incorporation of hCG priming injections might increase sperm yield.

Limited data exists on the long-term effects of regular hormone stimulation on physiological response in the anurans. Changes in sensitivity at the level of the gonads may be of great physiological significance (Rastogi et al., 2011). Gonadotropins act on the testis via receptors and the presence of various cell types within the same compartment requires coordination of cell functions to facilitate the production of spermatozoa (Pierantoni et al., 2002). Regular stimulation and subsequent desensitization
of hCG receptors through overuse of hCG might reduce the spermiation response to exogenous hormone treatment. Continued hormone treatment with gonadotropin hormone-releasing factor has been demonstrated to cause desensitization of the pituitary in other vertebrate, including anurans (Gallo et al., 1980; Rastogi et al., 2011). Overstimulation might initiate an immune response resulting in the production of antibodies that render the hormone ineffecual; in mammals this can occur after just 2-3 injections (Swanson 1995). However, unpublished data by Kouba et al. (2012) reported the regular use of hCG over a number of years in A. fowleri with no apparent physiological effects on spermiation response. Similarly, Waggener and Carrol (1998) reported no indication of a refractory response in L. laevis when males were allowed a recovery period of 30 days following administration of GnRH. The males used in this present study were given a 2-month period of recovery between hormone injections but there may be a substantial period of post-reproductive refractoriness in some anuran species (Rastogi et al., 2011). McDonough et al, (2015) demonstrated a reduced capacity for spermiation in A. fowleri injected with hCG twice a week for 5 weeks, and sperm depletion was reported in Bufo marinus injected twice a week with GnRHa (Roth and Obringer 2003). However, rather than an immunological-based response, it is thought more likely that down-regulation of GnRH receptors is responsible for the reduction in sperm production (Kouba and Vance, 2009). Therefore, although the collection of sperm through hormone therapy has an important role to play in the application of ARTs, it may confer detrimental outcomes on sperm quality, concentration and hormone receptivity and should be applied with caution if given frequently.
The boreal toad is a temperate seasonal species, breeding once a year between mid-May to mid-July (Hammerson, 1999). Seasonality was not addressed in this study, and the extent to which maintaining an active non-hibernated toad colony that retains the capacity to respond to hormone administration several times annually, requires further exploration. Biesinger and Miller (1952) demonstrated a reduced response to hormone stimulation of male amphibians outside of their natural breeding season. Environmental variables condition the amphibian’s physiological state and influence the reproductive activity of seasonally producing male toads by inducing spermatogenic activity (Rastogi, 2011). Therefore, it would be beneficial to establish the importance of seasonality on the reproductive response to exogenous hormone treatment in the boreal toad. An important consideration is that the boreal toad naturally undergoes an annual period of hibernation in the wild (Loeffler, 2001). The MSU toad colony was not hibernated for the first two consecutive winters after arrival at our animal facility, and this may have affected reproductive function. A period of winter dormancy is thought to influence reproductive output in anurans (Tsai, 2011). Calatayud et al., (2015) demonstrated that hibernation plays a key role in the reproductive behavior of female boreal toads from the same colony that was used in the present study. In the absence of hibernation, no female boreal toads oviposited in response to exogenous hormone treatment, in contrast to 77% of hibernated females which were induced to oviposit following administration of the same hormone treatment (Calatayud et al., 2015). Similarly, Roth et al. (2010) reported that hibernation in addition to hormone induction was required for gamete release in the female boreal toad.
Traditionally, the NASRF breeding protocol for the boreal toad involved injecting both the male and the female toads simultaneously with a combined hormone treatment of hCG and GnRHa [NASRF staff, pers. comm.]. Calatayud et al. (2015) recommends injecting female toads with two priming injections of hCG 96 h apart, followed by a combination ovulatory dose of hCG and GnRH 24 h later the second priming dose. The average time to oviposition was observed to be $36 \pm 6$ h post-ovulatory dose. Therefore, to synchronize gamete deposition for the purposes of AF in the boreal toad, males should be injected at least 34 h after the females receive an ovulatory dose in order to coincide with peak sperm response and maximize reproductive output. Despite the historical use of exogenous hormones at NASRF, the spermiation response to a combined hormone treatment (hCG + GnRHa) has not been quantified. Although, the simultaneous injection of male and female toads does induce amplexus, the spermiation response might not coincide with oviposition, i.e. peak sperm production may occur before or after egg deposition. Therefore it would be worthwhile characterizing the spermiation protocol of the boreal toad in response to the combined hormone treatment, to facilitate hormonally-assisted natural mating in this CBP.

Exogenous hormone therapy affords a number of practical benefits to a CBP including the capacity for repeated sperm collections from the same male which allows multiple paternities from one egg mass, through AF (Kouba et al., 2009). If exogenous hormone treatments are not optimized then reproductive output will remain sub-optimal. This is a potential problem if the lack of a reproductive response to a specific hormone treatment labels the hormone non-functional, where the case may simply be that the most effective dose has not yet been administered (Kouba et al., 2012). Hormone efficacy
trials should be encouraged in breeding facilities where there are accessible research animals so that the most effective dose for gamete induction can be established. Data from the present study is useful in the collection of sperm for AF purposes but further investigation is required to determine whether this hormone regimen would be applicable to assisted natural mating.

**Study 2: Short-term cold storage of hormonally-induced spermic urine**

Data from the cold-storage study demonstrated the capacity of *A. b. boreas* spermatozoa to retain motility after storage at 4°C for up to 14 days. However, duration of time in cold storage did have a detrimental effect on the recovery of spermatozoa motility parameters. After 14 days in cold storage, boreal toad spermatozoa motility had decreased by 60%, and the proportion of motile spermatozoa exhibiting forward progressive motility decreased significantly after 24 h and remained low (less than 10%) until day 12 when spermatozoa FPM ceased. Browne *et al.* (2001) demonstrated that testicular sperm from the cane toad maintained motility for longer when stored at 0°C in contrast to samples stored at 4°C. Therefore, it would be worthwhile exploring whether a lower storage temperature might increase sperm longevity in the boreal toad, although logistically this is much more difficult to manage over two weeks.

There are only a handful of studies that address cold-storage in amphibian spermatozoa, yet the use of cold-storage techniques are quite common in fish (Holt *et al.*, 1996). The ability to extend the longevity of spermic urine suggests that the metabolic rate is lowered and thus, energetic reserves are maintained over time (Browne *et al.*, 2001). Several studies in fish have indicated that build up in bacteria and the use of gentamycin inhibits bacterial growth (Dorsey *et al.*, 2011; Gorman *et al.*, 2011). Two
techniques commonly employed in aquaculture to extend the sperm longevity of cold stored sperm samples are aeration and antibiotic supplementation. The issue of toxins and bacteria was addressed by Germano et al. (2013) and Silla et al. (2014), with both studies indicating aeration was an effective method for increasing the longevity of sperm samples in cold-storage but reported an inhibitory effect of antibiotic supplementation on sperm motility. The addition of streptomycin had a detrimental effect on cold-stored sperm viability in the Fowler’s toad (Anaxyrus boreas boreas) and gentamicin-supplemented spermic urine samples exhibited significantly decreased motility in the booroolong frog (Litoria booroolongensis) (Germano et al., 2013; Silla et al., 2014).

Cold-stored (5°C) spermic urine samples of the booroolong frog aerated with 100% or 20% oxygen exhibited consistently higher proportions of motile spermatozoa than samples supplemented with gentamicin; however, a comparison of oxygenated samples to non-oxygenated and non-supplemented samples in this study was not described (Silla et al., 2014). Germano et al. (2013) showed that aerated Fowler’s toad sperm samples retained significantly greater motility (70%) during the first 24 h after collection than antibiotic-supplemented or non-supplemented and non-aerated control samples. However, Browne et al. (2001) found no effect of aeration on cold-stored sperm samples of the cane toad, indicating the potential for species-specificity in the tolerance of anuran sperm to short term cold-storage.

One proposed hypothesis is that spermic urine maintained in short-term cold storage might accumulate toxic metabolites that affect fertilization capacity and that premature activation of cold-stored sperm may also affect fertilization rates (Browne et al., 2001). Therefore, AF trials would be a valuable next step in validating spermatozoa
cold-storage protocols for the boreal toad. Anuran sperm is immotile in the testis and is activated by hypotonic environmental conditions related to spawning (Duellman and Trueb, 1994). As such, spermic urine samples maintained in cold-storage are stored in a naturally-activated state (Duellman and Trueb, 1994; Kouba et al., 2003). The extension of spermic urine samples in a high osmolality diluent that deactivates motility might be worthwhile exploring in the boreal toad. Browne et al. (2001) stored cane toad sperm samples in a 1:1 and 1:5 concentrated dilution and demonstrated that fertilization rates were significantly improved and fertilization capacity was maintained up to 10 days post-collection using sperm deactivated in a 1:5 dilution.

Further opportunities for increasing genetic diversity through spermatozoa cold-storage techniques include the transport of sperm samples between breeding facilities. Kouba et al., (2011) demonstrated that sperm samples could successfully be transported overnight to a different breeding facility in cold storage and maintain fertilization capacity (46% fertilization rate in Lithobates sevosa), representing a milestone in ART development for anurans. Cold-storage also provides the potential means to collect and store samples from free-living anuran populations. Gamete collection in the field for introduction into CBPs to increase genetic diversity is commonplace in the aquaculture industry, however, to date, there are no reports of this technique for anuran captive breeding.

**Conclusion**

Exogenous hormone protocols for gamete induction are a valuable addition to CBPs by facilitating the coordination of AF and assisted natural mating. Hormone efficacy and optimal dosages are being explored in an increasing number of captive
species in which natural mating is unreliable or absent. To date, spermiation response to hormone treatment has not been fully characterized in the boreal toad. The advantages of a reliable sperm collection protocol also extend to increasing longevity of sperm motility through short-term cold-storage, thereby increasing the window of opportunity to achieve AF. The present study investigated the 24 h spermiation response to three exogenous hCG dose treatments. Overall, the results from this study verified that spermiation could be successfully induced by administration of exogenous hCG. While the concentration of motile sperm and the quality of spermatozoa motility parameters did not differ significantly between hCG dose treatment, a greater number of males in the Medium and High treatment groups responded to hormone stimulation; therefore, based on a minimal effective dose, it would be pertinent to recommend administration of 10 IU g\(^{-1}\) as the most effective protocol for inducing sperm release in the boreal toad.

The ability to maintain viable samples of spermic urine at cold temperatures for several days would further enhance AF techniques and the reproductive output of CBPs. There are a number of practical benefits of using cold-storage techniques, including the facilitation of gamete synchronization since egg release in the anurans cannot always be reliably timed. Anuran eggs are only penetrable for a short period of time following oviposition and the jelly layer hardens and sperm cannot enter (Olson and Chandler 1999), a natural mechanism thought to prevent polyspermy. If spermic urine samples can be refrigerated over several days this increases the opportunity for successful AF. Moreover, multiple sperm samples could be collected from a single male, stored over several days, and used to fertilize several egg masses, facilitating additional paternities from a single collection period. This is especially helpful for very small populations,
where one male could potentially fertilize many females, rather than just a single mate pairing, allowing for rapid diversification of population genetics.

Recommendations for further refinement of the boreal toad spermiation protocol outlined in this study include: 1) quantifying the spermiation response to a combined hormone treatment of hCG and GnRHa; 2) investigating the potential to enhance spermiation response through administration of priming doses of hCG; 3) establishing the importance of hibernation on spermiation response to hCG treatment; and, 4) exploring the potential for the transportation of sperm samples between breeding facilities and the collection and storage of samples from wild populations into captivity.

Acknowledgements

This study was supported by an Institute of Museum and Library Services (IMLS) National Leadership Grant (LG-25-09-0064-09). The authors are grateful to the Mississippi Agricultural and Forestry Extension Service (MAFES) and Colorado Parks and Wildlife, particularly Harry Crockett, and Ted Smith and staff at the Native Aquatic Species Restoration Facility for providing the animals used in this study. We would also like to thank Michael Robinson and Lindsay Bullock for their assistance with the husbandry and care of the MSU boreal toad colony.
References


CHAPTER IV
SPERMIATION RESPONSE TO EXOGENOUS HORMONE THERAPY IN HIBERNATED AND NON-HIBERNATED SOUTHERN ROCKY MOUNTAIN BOREAL TOADS (ANAXYRUS BOREAS BOREAS)

Introduction
The life-history of anurans is strongly influenced by the external environment including weather and climate, with temperature of particular importance (Corn, 2005). Reproductive behavior, specifically, is characterized by a complex synergistic relationship between a suite of environmental stimuli and the neuroendocrine responses that regulate gonadal activity and breeding (Corn, 2005; Tsai, 2011). Reproductive cues vary depending on species and geography and include factors such as rising temperatures, rainfall, snowmelt, humidity, photoperiod, barometric pressure and food availability (Brizzi and Corti, 2006; Mann et al., 2010; Rastogi et al., 2011).

Temperature is a major influence on high elevation temperate species inhabiting seasonally extreme environments (Duellman and Trueb, 1994; Rastogi et al., 2011). In particular, gonadal growth and spermatogenesis are associated with temperature changes, tending to increase with rising temperatures and become depressed as temperature drops (Rastogi et al., 2011). The life-cycle of all temperate anuran species is characterized by seasonal breeding once a year, undergoing a single cycle of gametogenesis and breeding,
and annual migration of reproductive adults between breeding and hibernating habitats (Hartel et al., 2007; Rastogi et al., 2011). Reproductive patterns are strategically timed whereby mating, egg deposition and larval development are carefully synchronized with optimal environmental conditions (Hartel et al., 2007). This is important for maximizing reproductive output and population recruitment. Temperature can affect the time it takes for tadpoles to successfully complete metamorphosis before their natal pond freezes over with the onset of winter (Corn, 2005).

Northern temperate anuran species enduring extreme winter conditions face a number of challenges and a period of dormancy is a common strategy for survival (Storey and Storey, 1992). Hibernation provides a means to circumvent limited food availability and reduce the energetic costs of maintaining body temperature during seasonally cold periods (Storey, 2000). Metabolic rate is reduced well below normal resting state as a defense against harsh environmental conditions, allowing animals to remain in a hypometabolic or dormant state for several months at a time (Storey, 2000). Hibernation is also thought to play an influential role in reproductive function (Tsai, 2011). However, there is little information on the underlying physiological mechanisms involved.

Most northern climate anuran species employ terrestrial hibernation by overwintering in a hibernacula below the frost-line, as exhibited by a number of the Bufonidae, including the Southern Rocky Mountain (SRM) boreal toad (*Anaxyrus boreas boreas*) (Pinder et al., 1992; Zug et al., 2001). The SRM boreal toad is the only high elevation anuran species inhabiting the alpine and subalpine regions between 2300 and 2700m in Colorado’s Rocky Mountain National Park, (Loeffler et al., 2001). The life history of high elevation seasonal breeders, such as the boreal toad, is characterized by a
long annual period of dormancy (up to eight months); emerging to breed, typically between mid-May and mid-July (Hammerson, 1999).

The SRM boreal toad is currently a target population for restoration efforts due to devastating population crashes at many wild breeding sites in its native Rocky Mountain National Park (Carey, 1993). Over 600 SRM adult boreal toads are housed at the Colorado Parks and Wildlife’s Native Aquatic Species Restoration Facility (NASRF), in Alamosa, CO, and undergo annual seasonal breeding operations [NASRF staff. pers. comm.]. The entire colony undergoes an annual eight-month period of artificial hibernation prior to seasonal breeding operations. However, the boreal toad rarely breeds naturally in captivity and exogenous hormone treatments are required to initiate breeding behavior [NASRF staff, pers. comm.]. Reproductive dysfunction is a common problem in captive anurans where endogenous and environmental stimuli are often missing (Kouba et al., 2009) and despite the best efforts at NASRF to simulate a natural environment through a number of measures, including a period of hibernation, reproductive output remains low (Calatayud et al., 2015).

Hibernation can be a limiting factor in a captive breeding program by restricting reproductive events to a confined period. Furthermore, many breeding facilities may not be equipped to hibernate all species that possess this life-history characteristic. If this period of dormancy can be circumvented through the use of exogenous hormone stimulation for gamete production, the breeding window of seasonal species could potentially be extended and reproductive output increased. Maintaining a colony in an active state also eliminates any risk of hibernation-associated mortality. Hibernation can affect both the immune system and the gastrointestinal bacterial fauna, resulting in
immunosuppressed individuals with an increased susceptibility to infectious disease (Wright and Whitaker, 2001). Furthermore, simulated indoor hibernation requires reliable refrigerators and regular monitoring to avoid ‘freezer burn’ or desiccation resulting from inappropriate artificial hibernation techniques, which can result in mortality (Wright and Whitaker, 2001). The boreal toad breeding colony at NASRF lose, on average, 20 individuals per year through hibernation, which equates to around 3% of the colony [N. Calatayud, pers. comm.] and Roth et al., (2010) reported 2 mortalities out of 200 hibernated boreal toads. In large breeding populations, such a loss might be negligible but could pose a serious threat to small captive colonies. As such, breeding facilities may be disinclined to employ hibernation techniques that could potentially have serious consequences for small or rare populations.

Therefore, in some circumstances it might be advantageous to avoid hibernation if there is no clear effect on reproductive output. Since hibernation is known to play an important role in gonadal development and gamete maturity in a number of other vertebrate species, further investigation of the significance of anuran life history strategies, such as hibernation, is warranted. A small number of studies indicate the importance of hibernation for the induction of reproductive behavior, including a study on male boreal toads. Roth et al. (2010) recommends hibernating male boreal toads and inducing breeding behavior with exogenous administration of the hormone gonadotropin releasing-hormone agonist (GnRHa) to enhance breeding success; however, spermiation was not quantified in this study. As an increasing number of species with diverse reproductive strategies are brought into captivity, a more comprehensive understanding of amphibian endocrinology and the functionality of specific life-history traits will be
paramount to their successful *ex-situ* management (Clulow, *et al*., 2014; Roth and Obringer, 2003).

To this end, a sub-population of boreal toads, were transported from NASRF to an animal facility at Mississippi State University (MSU) for research and development of assisted reproductive technologies (ARTs) to enhance captive breeding efforts. Over the last several years of research, hormone protocols have been developed to induce gamete release and breeding behaviour in the boreal toad in order to optimize reproductive potential and maximize reintroduction efforts (Calatayud, *et al*., 2015; Kouba *et al*., 2009; Langhorne *et al*., unpublished). Exogenous administration of human Chorionic Gonadotropin (hCG) stimulates a spermiation response in the boreal toad that last up to 24 h (Kouba and Vance, 2009; Langhorne, unpublished). However, this was investigated in a non-hibernated population of boreal toads. In contrast to the boreal toads at NASRF, the MSU colony was maintained in an active state at room temperatures year-round; thus, is an excellent model to ascertain the significance of this life history trait on reproductive output.

Specifically, we will quantify and compare the spermiation response of boreal toads that undergo an annual cycle of hibernation, synchronous to wild conditions, with boreal toads deprived of hibernation for two consecutive breeding seasons.

**Materials and Methods**

All animal procedures were conducted following review and approval by the Mississippi State University Institutional Animal Care and Use Committee
Animals

All adult male boreal toads originated from egg masses collected in the southern Rocky Mountains or captive-reared at NASRF. Male boreal toads used in this study were from a designated research population that were not part of the captive breeding program. In October 2010, males \( n=21; 6-12 \text{ years old} \) were transferred from NASRF to Mississippi State University (MSU) and males \( n=19; 6-14 \text{ years old} \) were maintained at NASRF for comparison purposes. Toads received at MSU were not hibernated for the subsequent two breeding seasons, Toads maintained at NASRF remained on an annual hibernation regimen.

Non-hibernated boreal toad colony (NASRF)

The MSU colony of boreal toads were housed in ventilated polycarbonate containers \((30 \times 46 \times 66 \text{ cm})\) with access to water and shelter. Frogs were maintained between 20–23°C and provided with standard fluorescent lights on a seasonal light cycle. Single-sex groups of 4-5 individuals were housed per container and offered a variety of food items including crickets, wax worms, and mealworms, three times per week. Crickets were gut-loaded with Repashy SuperLoad© supplement (Repashy Ventures Inc., CA, USA) and dusted with Reptivite © powder (ZooMed Laboratories, Inc., Costa Mesa, CA, USA) prior to being fed out.

Hibernated boreal toad colony (MSU)

Prior to hibernation, male boreal toads \( n=19 \) housed indoors at NASRF were maintained on a natural light cycle in large fiberglass tanks \((121 \times 60 \times 30 \text{ cm})\) with a continuous supply of fresh water. Toads were fed a variety of prey items three times per
week, including gutloaded (BugBurger, Allen’s Repashy, La Jolla, California, USA) 
crickets, mealworms, red-wiggler worms and waxworms. Crickets were dusted with 
Reptivite © powder (ZooMed Laboratories, Inc., Costa Mesa, CA, USA) prior to being 
fed out. In mid-December 2012, toads were allocated 4-5/tub into ventilated plastic 
containers (33 x 13 x 15 cm) with a substrate of sand and sphagnum moss and placed into 
a refrigerator cabinet (Foster refrigerator, Corp., Hudson, New York, USA). Hibernacula 
temperatures ranged from 2-6° C. The toads were removed from hibernation in mid-May 
after a period of 5 months. This process was repeated on a yearly basis.

**Comparison of spermic urine production between hibernated and non-hibernated boreal toads**

The goal of this study was to compare sperm quantity and quality between two 
treatment groups: hibernated and non-hibernated male boreal toads, following exogenous 
hormone therapy. Boreal toads from the two treatment groups (MSU non-hibernated, \( n =21 \) vs. NASRF hibernated, \( n=19 \)) were administered an intra-peritoneal injection of 10 
IUg\(^{-1}\) body weight (BW) hCG and urine samples collected and analyzed for the presence 
of sperm as described below. If sperm was present in a given sample at a given time-
point, the individual was classified as a ‘responder’. Individuals not producing sperm at a 
given time-point were classified as ‘non-responders’ and were not included in any 
subsequent analysis. Spermiation response was then compared between MSU non-
hibernated males and hibernated males from NASRF. For the duration of the hormone 
trial, male boreal toads were maintained in plastic containers (35 x 20 x 13 cm) holding 
aged tap water (2 cm depth) to ensure continuous urine production. Prior to hormone 
administration, toad weight and snout-vent length (SVL) were recorded and a urine
sample (T₀) was obtained from each frog to determine the presence or absence of sperm. To collect urine, males were gently removed from their holding containers and held above a 150 mm petri dish, spreading the hind limbs apart by the thumb and index finger, until urination occurred (usually within 1 min).

Spermic urine was collected at 2, 3, 5, 7, 9, 12 and 24 h post-hormone administration (PA). A control group of males (n=10) was injected with 200 µl of sterile saline. Samples were analyzed immediately post-collection by placing a 10 µl aliquot of spermic urine onto a glass slide under 400x objective on an Olympus CX41 phase-contrast microscope and counting the first 100 sperm. Spermic urine variables measured included volume, percent sperm Total Motility ([TM] = flagellum movement), percent Forward Progressive Motility ([FPM] = forward moving sperm as a proportion of total count), and FPM Quality ([FPM-Q] = a subjective scale from 0 (no movement) to 5 (very rapid forward movement). Sperm concentration was measured by inactivating motility in a 1:10 dilution of PBS and counting on a Neubauer haemocytometer to obtain an average sperm concentration mL⁻¹.

**Statistical analysis**

Assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk and Levene’s tests, respectively. Body parameters (weight and SVL) and the number of male responders were compared between hibernated and non-hibernated groups using an independent samples \( t \)-test. Sperm parameters (Concentration, Total Motility [TM], Forward Progressive Motility [FPM] and FPM-Quality [FPM-Q]) was compared between hibernated and non-hibernated toads by a two-way repeated measures analysis of variance (ANOVA) using the General Linear Model procedure (GLM). A
split-plot model was used to analyze the effect of hibernation on spermiation response across time (0, 2, 3, 5, 7, 9, 12 and 24 h PA) where time was the main plot factor and treatment (hibernation, non-hibernation) the sub-plot factor. Within the model, treatment and time were fixed factors, and sperm parameters (Concentration, TM, FPM and FPM-Q) were dependent factors. Individual frog was nested within treatment to remove variation among individuals from the error term. Significant main effects were explored using Tukey-Kramer Honestly Significant Difference (HSD) post-hoc tests. Percentage data were arcsine transformed using the transformation \(\sin^{-1}(\sqrt{x})\) before further analysis. All values are expressed as mean ± SEM and significance was established at \(P \leq 0.05\). All statistical analysis was performed in SAS Version 9.4 (Cary, North Carolina, USA).

Materials and Methods

All animal procedures were conducted following review and approval by the Mississippi State University Institutional Animal Care and Use Committee.

Effects of hibernation on spermiation response

Male boreal toads that underwent a period of hibernation prior to exogenous hormone stimulation weighed significantly more \((t_{23}=4.30; P<0.001)\) than non-hibernated toads \((47.9 \pm 2.5 \text{ g and } 35.1 \pm 0.9 \text{ g, for hibernated and non-hibernated groups, respectively,})\) but mean SVL did not significantly differ \((t_{31}=; P=0.12)\) between the groups \((64.1 \pm 1.1 \text{ mm and } 61.5 \pm 0.7 \text{ mm, for hibernated and non-hibernated groups, respectively})\). Urine samples were successfully collected from 100% males at each collection time-point (2, 3, 5, 7, 9, 12 and 24 h PA). All urine samples collected prior to
hormone treatment (To) were aspermic. No animals in the control groups receiving a sterile saline injection only, produced sperm

**Spermiation response**

Responding male toads were classified as individuals producing spermic urine at a given time-point PA, and non-responders as males with no spermatozoa present in a urine sample for the corresponding time-point (2, 3, 5, 7, 9, 12 and 24 h PA). A spermiation response was induced in >60% of hibernated and non-hibernated males across all time-points within 2 h PA, following exogenous hormone administration of 10 IU g⁻¹ hCG (Figure 11). There was no significant difference ($t_{38} = 4.90; P>0.05$) in the number of males responding to hormone treatment between hibernated and non-hibernated toads (Figure 11). The spermiation response remained between 74%- 84% of males across the collection period, with the exception of a decrease to 57% of non-hibernated males producing spermic urine at the 24 h collection period (Figure 11).
Urine was collected from hibernated (n=19) and non-hibernated (n=21) male boreal toads at seven different collection time-points (2, 3, 5, 7, 9, 12 and 24 h) post-hormone administration (PA) of a 10 IU g⁻¹ dose of hCG. Responders were classified as individuals producing spermic urine at each time-point PA. A spermiation response was initiated within 2 h PA in both hibernated and non-hibernated toad groups. Male response to exogenous hormone treatment did not significantly differ between hibernated and non-hibernated toad groups across all 7 collection time-points in the 24 h collection period (P>0.05). Values are expressed as percentages of the total number of males responding in each treatment group [hibernated vs non-hibernated] within a time-point.

**Sperm concentration**

Sperm concentration of male boreal toads was significantly higher in the hibernated male toad group, compared to the non-hibernated male group (F₁,₁₆₁=49.6; P<0.001; Figure 12). Furthermore, sperm concentration varied according to different time-points following exogenous hCG administration (one-way ANOVA; F₆,₁₆₁=3.81; P<0.001). Peak sperm production was between 3–7 h PA when significantly higher sperm concentrations were released than at any other collection time-point (Tukey-
Kramer; $P<0.001$). Figure 12). Specifically, peak sperm concentrations were $4.3 \pm 0.8 \times 10^6$ mL$^{-1}$ to $5.2 \pm 1.3 \times 10^6$ mL$^{-1}$ and $1.5 \pm 0.6 – 1.8 \times 10^6$ mL$^{-1}$, for hibernated and non-hibernated boreal toads, respectively. Sperm production was significantly depleted by the 24 h collection period with $1.7 \pm 0.5 \times 10^6$ mL$^{-1}$ and $0.6 \pm 0.3 \times 10^6$ mL$^{-1}$ for both hibernated toads and non-hibernated treatment groups, respectively (Tukey-Kramer; $P<0.05$; Figure 12). There was no significant treatment by time interaction with regards to sperm concentration (one-way ANOVA; $F_{6,161}=1.07; P=0.38$), indicating that the direction and general spermiation profile between both groups [hibernated vs. non-hibernated] did not differ across time (Figure 12).

![Figure 12](image)

Figure 12  Mean sperm concentration released by hibernated and non-hibernated boreal toads over a 24 h period in response to administration of 10 IU g$^{-1}$ hCG

Urine was collected from hibernated ($n=19$) and non-hibernated ($n=21$) male boreal toads at seven different time-points (2, 3, 5, 7, 9, 12 and 24 h) post-hormone administration (PA) of 10 IU g$^{-1}$ hCG. Responders were classified as individuals producing spermic urine at each time-point PA. A spermiation response was initiated within 2 h PA. Hibernated male toads produced significantly higher sperm concentrations across the collection time. ($P<0.05$). Values are expressed as means of the total number of male responders exhibiting motile sperm in each treatment group [hibernated and non-hibernated] at each collection time-point ± SEM.
Figure 13.A shows that the general pattern of spermatozoa TM remained relatively constant over the 24 h collection period, ranging between 87.4 ± 1.6% to 89.7 ± 1.3% and 59 ± 4.5% to 76 ± 2.4% in the hibernated and non-hibernated treatment groups, respectively. There was no significant difference ($F_{6,131}=1.48; P=0.22$) in the proportion of motile sperm released by hibernated and non-hibernated *A. b. boreas* and no treatment by time interaction was observed ($F_{6, 131}=1.11; P=0.35$; Figure 13.A). There was, however, a significant effect of time ($F_{6, 131}=3.11; P<0.01$), with higher proportions of motile sperm between 3- 12 h PA, compared to 24 h PA (Tukey-Kramer; $P<0.05$; Figure 13.A).

Figure 13.B. shows the proportion of FPM sperm released by hibernated and non-hibernated males over 24 h in response to hormone treatment. There was a significant treatment by time interaction ($F_{6, 127}=3.43; P<0.01$; Figure 13.B), indicating that the proportion of forward motile spermatozoa and direction of response varied over the 24 h collection period between the hibernated and non-hibernated toad groups (Figure 13.B.). Specifically, spermatozoa FPM within the hibernated male group was significantly higher across all collection time-points, with the exception of 24 h PA, peaking rapidly at 3 h PA with 72 ± 3.1% of spermatozoa exhibiting forward motility ($P<0.001$). The proportion of FPM steadily decreased, in hibernated toads, by 7- 9 h PA with 54.7 ± 4.5% at 7 h and 57 ± 4.5% at 9 h, respectively, and finally decreasing to 34 ± 7.1% by 24 h PA (Figure 13.B.). In contrast, sperm collected from non-hibernated males was significantly lower and demonstrated a different pattern across time, FPM steadily increasing between 2 and 7 h PA with a rate of 49 ± 4.5% at 7 h before steadily decreasing to 24 h PA with a rate of 23 ± 5.1 % FPM (Figure 13.B.). Consistent with spermatozoa FPM, there was an overall
interaction effect of treatment over time in the quality of spermatozoa FPM [FPM-Q] released by hibernated and non-hibernated males ($F_{6, 127}= 2.15; P=0.05$; Figure 14).

Figure 13 Motility parameters of spermatozoa released by hibernated and non-hibernated boreal toads over a 24 h period in response to administration of 10 IU g⁻¹ hCG

Mean percentage of (A) Total Motility (TM) and (B) Forward Progressive Motility (FPM) of urine samples collected from hibernated ($n=19$) and non-hibernated male boreal toads ($n=21$) at 7 time-points (2, 3, 5, 7, 9, 12 and 24 h) post-hormone administration (PA) of a 10 IU g⁻¹ hCG. Spermatozoa TM did not significantly differ between hibernated and non-hibernated toad groups ($P>0.05$) but was significantly higher between 3–12 h PA ($P<0.05$). Significantly higher proportions of forward motile sperm were produced by hibernated male toads, relative to non-hibernated toads across the 24 h collection period ($P<0.05$). Values are expressed as untransformed means of the total number of male responders exhibiting motile sperm in each treatment group [hibernated vs non-hibernated] at each collection time-point ± SEM.
Mean FPM Quality (FPM-Q) of urine samples collected from hibernated ($n=19$) and non-hibernated ($n=21$) male boreal toads at 7 time-points (2, 3, 5, 7, 9, 12 and 24 h) post-hormone administration (PA) of 10 IU g$^{-1}$ hCG. Spermatozoa FPM-Q of hibernated male toads was significantly higher than that of non-hibernated toads ($P<0.05$) and ranged between a ranking of 3-5 and 1-3 in hibernated and non-hibernated toads, respectively, across the 24 h collection period. Significantly ($P<0.05$) higher quality sperm was released between 5-12 h PA ($P<0.05$). Values are expressed as means of the total number of male responders exhibiting motile sperm in each treatment group [hibernated vs non-hibernated] at each collection time-point ± SEM.

Overall, hibernated male toads produced significantly higher quality sperm ($P<0.05$) with a significant difference in the quality of spermatozoa at specific collection time-points. For example, higher quality sperm was produced between 5–9 h PA (Tukey-Kramer; $P<0.05$; Figure 14) than other time-points. FPM-Q ranged between a rank of 1.2 ± 0.2 to 3.3 ± 0.3 in the non-hibernated males and a higher rank of 3.1 ± 0.5 to 4.5 ± 0.2 in the hibernated male toads (Figure 14).
Discussion

The objective of the present study was to ascertain the extent to which hibernation influences reproductive behavior by quantifying and comparing the spermiation response to hormone treatment of male boreal toads that typically undergo a yearly cycle of hibernation, synchronous to wild conditions, with boreal toads deprived of hibernation for two consecutive breeding seasons. The overall aim was to determine hormone therapy of non-hibernated toads could be used to circumvent natural reproductive cues for the stimulation of gametogenesis, in order to increase reproductive output of captive male boreal toads. These results clearly show that hibernation has a positive effect on reproductive output in captive boreal toads.

Effect of hibernation on spermiation response

Although hibernated, the boreal toad breeding colony at NASRF rarely display natural reproductive behaviors and over the last decade exogenous hormone treatments have been incorporated into the breeding management of the colony [NASRF staff, pers. comm.]. However, response to hormone treatment has been variable and low reproductive failure each breeding season compromises reintroduction efforts through the under-representation of valuable lineages. For obligate hibernating species, the breeding window in the wild is narrow and by emulating these natural conditions in captivity - through a five month artificial hibernation period - breeding opportunities are limited. If hibernation can be circumvented by exogenous hormone treatments to stimulate gamete induction, breeding operations could potentially be extended, thus increasing reproductive output.
The boreal toads at MSU were maintained in an active state year-round and, similar to the NASRF toads, did not exhibit natural breeding behavior, although both treatment groups did produce sperm in response to hormone therapy. Administration of 10 IU g\(^{-1}\) hCG initiated a spermiation response in both hibernated [NASRF] and non-hibernated [MSU] male boreal toads. The timing and pattern of the spermiation profile of both treatment groups were similar, peaking in sperm production at 5 h PA. However, hibernated males produced significantly greater concentrations of higher quality spermatozoa compared to non-hibernated toads. Specifically, hibernated toads at peak production produced almost four times the concentration of sperm released by non-hibernated toads (5.3 ± 1.0 x 10\(^6\) mL\(^{-1}\) and 1.5 ± 0.4 x 10\(^6\) mL\(^{-1}\), respectively). These results suggest that a period of hibernation is beneficial for maximizing overall reproductive output in the boreal toad through a higher sperm: egg ratio, leading to higher fertilization rates.

Male toads are physiologically primed for breeding in response to environmental variables that cue emergence from hibernation (Lofts, 1964). Consequently, the absence of stimuli in captivity may disrupt the natural pattern of spermatogenic activity, resulting in a reduced spermiation response. The boreal toad is a discontinuous breeder and, as with other temperate seasonal producing species, spermatogenesis occurs during or directly following the breeding season (Duellman and Trueb, 1994). Immature sperm is stored in the seminiferous tubules until the initiation of breeding when sperm cells mature homogenously throughout the testes, prior to release in urine (Duellman and Trueb, 1994; Rastogi et al., 2011).
Seasonal changes have been reported in the testicular morphology and function of a number of anuran species (Guarino et al., 1998; Kannamadi and Jirankali, 1992; Ko et al., 1998). The amplitude of the annual testicular cycle is greatest in anurans with discontinuous cycles, in contrast to species with continuous spermatogenic cycles (i.e. tropical species) that show no signs of seasonal fluctuations (Lofts, 1964). If toads are kept active year-round, as well as being subject to regular hormone treatment, the spermatogenic cycle might not arrest, and the testes could potentially contain a heterogeneous mixture of sperm cells at varying stages of development. This might explain the significantly reduced sperm quality of non-hibernated boreal toads. If hormonally-induced sperm, obtained outside the normal breeding season, is immature then a period of hibernation may be necessary to optimize reproductive response.

Calatayud et al. (2015) reported a significant correlation between hibernation with exogenous hormone treatment, and oviposition in female boreal toads. Specifically, hibernation alone did not induce spontaneous oviposition but in combination with hormone therapy the likelihood of eliciting a behavioral response was increased (Calatayud et al., 2015). Roth et al. (2010) demonstrated similar findings in an earlier study, but also reported successful oviposition of non-hibernated female boreal toads. Additionally, male reproductive behavior (i.e. initiation of amplexus) was significantly more prevalent in hormonally-stimulated boreal toads that had undergone a period of hibernation than hormonally-stimulated male toads kept active year-round (Roth et al., 2010). Development to tadpole from artificial fertilizations in both studies was low but it is unclear whether this was a result of poor egg quality, asynchronous gamete release, or a sub-optimal spermiation response (Calatayud et al., 2015; Roth et al., 2010). The
variability in reproductive response to hibernation and exogenous hormone treatment suggests that there may be other environmental factors responsible for the repeated breeding failure of both the hibernated NASRF boreal toad colony and the non-hibernated toads in the MSU colony.

Photoperiod and temperature, or a combination of both, are likely the primary proximate causes that convey information to temperate zone anuran species for predicting the optimal mating period (Saxena, 2009). It is widely reported that both water temperature and photoperiod are associated with spermatogenesis in a number of aquatic species (Lemaitre 2009; Borg, 1982). Photoperiod alone has been shown to affect ovarian development and gametogenesis in several frog species (Easley et al., 1979). Female and male bullfrogs (Rana catesbeiana) require a 12 / 12 h light/dark cycle for optimum ovarian maturation or maintenance of active spermatogenesis (Moore and Deviche, 1988), and the Japanese hylid frog (Hyla japonica) only initiates oocyte meiotic stages of division under a natural photoperiod (Wright and Whitaker, 2001). Photoperiod has also been shown to significantly affect the spermatogenic cycle in salamanders (Wright and Whitaker, 2001), and, in addition to temperature, is an important proximate cause in the stimulation of gonadotropin secretion and testicular activity in the edible frog (Rana esculenta) (Saxena, 2009). Though, for a number of other anuran species, photoperiod appears to have no significant effect on reproduction, suggesting that it is not a dominant stimulator of gonadal regression or recrudescence but might act to coordinate gametogenesis in some species (Moore and Deviche, 1998). In the photoperiodically-influenced R. esculenta, low ambient temperatures can slow or arrest spermatogenesis. However, only when ambient temperatures are elevated is spermatogenesis enhanced by
photoperiod (Lofts, 1964). This suggests that photoperiod is likely an important factor to consider in captive breeding, but is perhaps not exclusively influential on reproductive function and should be considered along with temperature in the husbandry regime of captive anurans. The MSU boreal toad colony was maintained on a standard annual light cycle of 12 / 12 h which, if the boreal toad is physiologically hard-wired to seasonality, may have negatively affected reproductive function, in addition, temperature might also play a significant role.

Since anurans are poikilothermic, it is not unexpected that gonadal activity is influenced by temperature (Moore and Deviche, 1998). Inappropriate temperature regimes in CBPs can affect reproduction, and thermal optimums for gametogenesis vary greatly between species, irrespective of geographic location (Rastogi et al., 201; Wright and Whitaker, 2001). In general, gametogenesis of continuous breeding anurans operates within a wide temperature range and at a higher thermal regime, relative to temperate species, to allow spermatogenesis year-round (Wright and Whitaker, 2001). In the South American toad (Bufo spinolosus) spermatogenic activity is decreased by lower temperatures which can cause degeneration of primary spermatocytes in the common green frog Hyla erythrea (Rastogi et al., 2011). Some species have a very small optimum temperature range in which spermatogenesis will occur, such as the Creole frog (Leptodactylus chaquensis) and the common green frog H. erythraea; both thermally sensitive to temperatures out-with a range of 20-24° C (Ce, 1980; Rastogi et al., 2011). The small optimum range of L. chaquensis is markedly different to the sympatric species, L. ocellatus, in which spermatogenesis occurs in a widespread optimum range of between 16-28° C, illustrating the species-specific nature of thermal sensitivity among the
anurans (Cei, 1980). In some seasonally-producing sub-tropical anurans, germinal cysts develop no further than primary spermatocytes but full spermatogenesis can be induced in an artificial environment by exposure to elevated temperatures (Wright and Whitaker, 2001). Temperate zone discontinuous breeders, such as the boreal toad, are unable to achieve this due to a post-breeding testicular refractory phase when testosterone levels are thought to decrease (Lofts, 1987).

Warming temperatures appear to stimulate luteinizing hormone (LH) release from the pituitary in *R. temporaria*, thereby preparing animals for spawning shortly after emergence from hibernation (Sotowaska-Brochocka and Licht, 1992). Studies on *R. esculenta* indicate that by early summer, estradiol inhibits the hypothalamic release of gonadotropin-releasing hormone (GnRH) and ultimately LH secretion, resulting in decreased testosterone levels and a seasonal refractory period in this species (Minucci et al., 1989). Anecdotal evidence from boreal toads used in the present study supports the existence of a post-breeding refractory period in the hibernated boreal toad, as several toads from this group did not spermiate in response to administration of the same hormone regimen eight weeks after the initial hormone treatment [pers. observ.]. However, this was not observed within the non-hibernated male group, which were allowed an eight week period of recovery between hormone injections.

The MSU boreal toad colony were housed in a laboratory facility where temperature could not be manipulated and as such, toads were maintained at 20-23°C year round, in addition to a water supply at room temperature. In contrast, the hibernated boreal toad colony at NASRF was maintained at a lower temperature range (15-18°C), with an external source of continually flowing water at natural fluctuating outdoor
temperatures. It is possible that the warmer environment exposed the MSU boreal toad colony to temperatures above their optimal thermal range for spermatogenesis, resulting in impaired sperm production. The use of a water chiller might be a necessary incorporation into CBPs for boreal toads and other high elevation seasonal frogs subject to continuous colder temperatures, particularly in facilities where room temperature cannot be adjusted.

The NASRF boreal toads weighed significantly more than the toads housed at MSU, therefore diet and body condition could also be contributory factors to the sub-optimal spermiation response of the non-hibernated boreal toad. Roth et al. (2010) reported that weight was a significant factor in the fecundity of hibernated female boreal toads. However, Calatayud et al. (2015) found no significant effect of weight on the likelihood of oviposition in A. b. boreas. Frogs and toads store their energetic supplies in abdominal fat bodies that can comprise several classes of lipid and protein and can synthesize steroid hormones in vivo (Rastogi et al., 2011). Fat body mass is directly liked to reproductive success and there are indications that, in both sexes, fat bodies are necessary to support normal gonadal activity (Rastogi et al., 2011). Androgens utilize lipids as their substrate and deplete the enlarged fat body cells at breeding season (Lofts, 1964). These histological changes are associated with the development of secondary sex characteristics and successful breeding behaviors (Lofts, 1964). Removal of fat bodies in female anurans negatively impacts the reproductive system by essentially shutting down vitellogenesis by impeding the transfer of essential lipids and proteins to pre-vitellogenic follicles (Pierantoni et al., 1983; Rastogi et al., 2011). In males, removal of fat bodies
might cause degeneration of primary spermatocytes and testicular atrophy (Rastogi et al., 2005).

The nutritional requirements of captive anurans are poorly understood (Ogilvy et al., 2012; Rastogi et al., 2011). Therefore, maintenance of reproductive condition through an appropriately nutritious diet is crucial for optimizing reproductive success of anurans in captivity (Kouba et al., 2012). The MSU boreal toad diet is very similar to the diet developed at NASRF including regularity of feeding (although boreal toads are not fed red wigglers). Hence, there may be other external factors affecting the body condition and, consequently, the reproductive function of captive boreal toads.

It is possible that the absence of a period of hibernation might function as a stressor to obligate hibernating species, thereby inhibiting normal reproductive physiology and behavior. The reproductive system is the first thing to shut-down when an animal is under stressful conditions and stress-induced reproductive failure is a common problem for many CBPs (Kouba et al., 2012). It is well established that stress increases cortisol levels in captive anurans and that this can inhibit critical endocrine pathways that regulate gametogenesis and breeding (Carr, 2011). A stress event causes the hypothalamic release of corticotrophin-releasing hormone which travels to the anterior pituitary where adrenocorticotropic (ACTH) hormone is released into the blood (Narayan, 2013). The ACTH release stimulates the release of glucocorticoids, specifically corticosterone in amphibians (Narayan, 2013). Elevated plasma levels of corticosterone can influence gametogenesis, sex steroid synthesis, and reproductive behavior (Carr, 2011). Regulating the stress response are a suite of physiological changes that have evolved to inhibit reproductive behavior when under threat (Narayan, 2013). A
major problem for captive breeding is that the physiological response to stress is induced by numerous stimuli, many of which are unknown, and, if prolonged, can chronically elevate glucocorticoids and influence the long-term reproductive capacity of captive animals (Carr, 2011).

A basic understanding of the life-history of a target species, including the requisite triggers for the regulation of gametogenesis and breeding, are fundamental to the successful outcome of captive breeding efforts (Rastogi et al., 2011; Monfort, 2014; Wright and Whitaker, 2001). In an ideal world, captive breeding populations would be located in the native range of the target species, allowing animals to be maintained within their natural environmental parameters (Pritchard, 1995). However, this is generally not a practical option for many species, and important reproductive cues are often missing, unknown or cannot feasibly be replicated in captivity (Kouba et al., 2009). Further examination of the neuroendocrine basis of amphibian reproduction is required for a more comprehensive understanding of how disruption of reproduction could occur under perturbed environmental conditions (Tsai, 2011). Thus, with an increasing number of species facing imminent extinction there is little time to investigate the mating cues of all species in need of rescue, presenting an enormous challenge for CBPs.

Exogenous hormone administration for gamete release is a mechanism by which spermatozoa can often be obtained outside the natural constraints of a breeding season. Therefore, the significance of natural stimuli, and to what extent they can be circumvented by exogenous hormone treatments are important factors to consider in the development of ARTs. The results of this study indicate that a period of hibernation is necessary to maximize the spermiation response of male boreal toads administered an
exogenous hormone treatment of 10 IU g\(^{-1}\) hCG. If hibernation is not an option within a CBP (either because it is deemed too risky, or resources are not available) a spermiation response, albeit at a significantly lower yield, can still be achieved in non-hibernated toads through the use of exogenous hormone administration. It may be the case that some species are so physiologically hardwired to hibernation that hormone therapies alone cannot produce optimal sperm production in non-hibernated males and concentrations will continue to be at a sub-optimal level. Under these circumstances, the provision of artificial hibernation, where possible, should be prioritized over the extension of the breeding season through hormone treatments.

**Conclusion**

For temperate annual breeding species, hibernation serves as a means to avoid extreme winter but it is also thought to play an influential role in reproductive function (Tsai, 2011). However, there is little information on the underlying physiological mechanisms and few examples in the literature relating to anuran hibernation. With an increasing number of species, characterized by diverse reproductive strategies, being brought into captivity, understanding the functionality of specific life-history traits will be paramount to their successful *ex-situ* management. The findings presented in this study illustrate the positive influence of a period of hibernation on the seasonal spermiation response of boreal toad; however, sperm could still be obtained outside of the normal breeding season, but at a lower quantity and quality. This information will be valuable for the captive breeding management of other threatened seasonal anuran species that would naturally hibernate in the wild.
Acknowledgements

This study was supported by an Institute of Museum and Library Services (IMLS) National Leadership Grant (LG-25-09-0064-09) and The Memphis Zoo’s Conservation Action Network award. The authors wish to thank the Mississippi Agricultural and Forestry Extension Service (MAFES) and Colorado Division of Parks and Wildlife, in particular, Harry Crockett, Ted Smith, Joe Marrinan, Tom Mix and all the staff at the Native Aquatic Species Restoration Facility for providing the animals used in this study and accommodating our research. We would also like to thank Michael Robinson, Lindsay Bullock, Christy Steadman and Andrew House for their help with the husbandry and care of the MSU boreal toad colony and Amanda Mullen for assistance with data collection.
References


CHAPTER V
SPERMATOZOA CRYOPRESERVATION OF THREE BUFONID SPECIES: THE FOWLER’S TOAD (ANAXYRUS FOWLERI), THE SOUTHERN ROCKY MOUNTAIN BOREAL TOAD (ANAXYRUS BOREAS BOREAS), AND THE PUERTO RICAN CRESTED TOAD (PELTOPHRYNE LEMUR)

Introduction
In recent years, Genetic Resource Banks (GRBs) have been promoted as a means to preserve the extant genetics of endangered species worldwide through the cryopreservation of gametes and tissue samples for long-term storage (Ryder, 2000). In addition to breeding and maintaining a live colony, amphibian captive breeding programs (CBPs) would benefit enormously from the ability to store gametes indeterminately, for future breeding efforts and the security of genetic diversity. In 2005, the International Union for the Conservation of Nature’s (IUCN) Global Amphibian Summit listed biobanking as one of its top 11 action steps to counter the global amphibian extinction crisis (Gascon et al., 2007), and several zoos and research institutions have established Genetic Resource Banks (GRBs) in the intervening years (Kouba., et al., 2013).

Gamete repositories are of enormous practical use in facilitating the genetic management of CBPs by: (1) safeguarding genetic diversity against loss through disaster or disease; (2) securing valuable founder genetics for future breeding, even after the death
of the donor individual; (3) facilitating the transport of genetics between breeding institutions, without compromising animal welfare or biosecurity; (4) providing a means to increase small population genetics without housing additional animals; (5) facilitating gamete synchronization for artificial fertilization through the year-round provision of gametes; and, (6) providing a potential resource for securing genetics from wild populations to rediversify captive populations (Frankham et al., 2010; Kouba and Vance, 2009a; Kouba et al., 2013; Wildt, 2000).

Amphibian biobanking is currently aimed at freezing sperm cells, and in the last two decades, cryopreservation and successful recovery of post-thaw motility of testicular sperm has been achieved in a number of frog and toad species, including: *Rana temporaria* (Kaurova et al., 2006; Mansour et al., 2010); *Bufo marinus* (Browne et al., 1998, 2001; 2002a); *Lithobates sylvatica* and *L. pipiens* (Beesley et al., 1998; Costanzo et al., 1998; Mungano et al., 1998); *Litoria brevipalmata, L. fallax, L. subglandulosa, L. leseuri*, *L. phylochroa, L. peroni, L. nausata, L. latopalmata, L. denata* and *Limnodynastes peroni, Crinia signifera, Philoria spp.*, and *Pseudophyrne bibroni* (Browne et al., 2002b); *Eleutherodactylus coqui* (Michael and Jones, 2004); and, *Xenopus laevis* and *X. tropicalis* (Buchholz et al., 2004; Mansour et al., 2009ab; Sargent and Mohun, 2005). However, to date, there is little data available on the successful application of cryopreservation techniques to threatened or endangered anuran species (Kouba and Vance, 2009a). Until recently a major hurdle in banking down valuable captive anurans was the nature of gamete collection, which required sacrificing the donor male to excise and macerate the testes (Kouba and Vance, 2009a; Kouba et al., 2013).
For species of conservation concern this is not a viable option, other than to opportunistically collect gametes from naturally deceased individuals.

The successful cryopreservation of sperm collected by non-lethal methods has since been reported in three different anuran species: *Rana pipiens* (Hopkins and Herr, 2007), *Rana temporaria* (Shishova et al., 2011), and *Pelophylax lessonae* (Uteshev et al., 2013). The technique employs exogenous hormone administration to stimulate spermiation, and the recovery of spermatozoa in urine (spermic urine), either by cloacal lavage (Hopkins and Herr, 2007), or abdominal massage (Shishova et al., 2011; Uteshev et al, 2013). The development was a major step forward in realizing a genetic resource bank for the collection and preservation of rare, declining and endangered anuran species. However, to date there are no further reports on the successful application of this novel technique to anuran sperm cryopreservation, particularly with regards threatened species.

The process of cryopreservation is a major stressor to spermatozoa, reducing both functionality and survival (Holt et al., 2003). There are two factors that govern cell cryopreservation (1) the freezing solution used to protect the cells from damage, and (2) the rate at which the cells are cooled. Successful cryopreservation requires the prevention of ice-crystal formation through the use of cryoprotectant agents (CPAs). CPA solutions protect cells from the freezing process and can be divided into two main types: permeating CPAs that penetrate the plasma membrane, or non-permeating CPAs that remain outside the cells. The function of the permeating cryoprotectant is to lower the freezing point of the solution so as to extend the time for cell dehydration and ensure that sufficient intracellular water exits the cell before freezing (Shaw and Jones, 2003). Permeating cryoprotectants can have differential toxicity depending on the type and time
of exposure, which can all affect the rate of cell penetration (Chian et al., 2010; Swain and Smith, 2010). Non-permeating cryoprotectants also function in cell dehydration during the cooling process, and in the prevention of osmotic swelling during thawing (Lahnsteiner et al., 1996).

There are several CPAs commonly employed across a wide range of taxa (Watson and Holt, 2001). The most frequently utilized for anuran sperm cryopreservation are the permeating CPAs dimethyl sulfoxide (DMSO), glycerol, and more recently N, N-dimethyl formamide (DMFA), and non-permeating CPAs, typically monosaccharide or disaccharide sugars, such as sucrose and trehalose, and proteins and lipoproteins (e.g. milk, egg yolk). The protective effect of a combination of cryoprotectants can be greater than would be expected if the action of each agent were simply additive (Chian et al., 2010), therefore cryoprotectant solutions often contain both permeating and a non-permeating CPAs. Two important procedures related to permeating cryoprotectants are the addition of a cryoprotectant to a cell before freezing and the removal of the cryoprotectant after freezing and thawing, both of which can cause significant osmotic stress that might damage the cell (Gao, et al 1995). Therefore, the first step in the development of a cryopreservation protocol is selection of the most suitable CPA based on sperm motility and viability recovery following a period of equilibration in the cryoprotectant solution.

There are two freezing methods employed in the cryopreservation of gametes: slow-rate freezing and ultra-rapid freezing (vitrification). Slow-rate freezing is typically employed in anuran sperm freezing and utilizes a freezing rate <40° C min⁻¹ and CPA concentrations in the range of 5% to 15%, whereas vitrification employs cooling rates of
>1000°C min\(^{-1}\) and CPA concentrations in the range of 40% to 60% (Cuevas-Uribe \textit{et al.}, 2011; Lahnsteiner \textit{et al.}, 1996). An inverse relationship exists between cooling rate, and the concentration of cryoprotectant (i.e. the faster the cooling rate, the higher the concentration of cryoprotectant needed and \textit{vice versa}) (Mazur \textit{et al.}, 2008). A slow cooling rate slows the process of water removal from the cell such that an equilibrium exists between the water content inside and outside the cell, preventing the formation of intracellular ice crystals (Watson and Fuller, 2001). The seminal studies of Luyet and Hodapp (1938) utilized vitrification for the successful cryopreservation of frog spermatozoa. Indeed the majority of gamete cryopreservation research of the early 20\textsuperscript{th} century employed this freezing technique. However, vitrification was supplanted by slow-rate freezing techniques with the discovery of glycerol as an effective cryoprotectant in 1949 (Polge \textit{et al.}, 1949).

Since Barton and Guttman in 1972, reported the successful cryopreservation of \textit{B. americanus} sperm using slow-rate cooling, sperm cryopreservation has been attempted on about 20 common anuran species. However, species-specificity exists in cell tolerance to cryodamage sustained during the freezing process, as such, gamete cryopreservation techniques often require modification at a species level (Holt, 2000). It seems unlikely, given the vast diversity in reproductive modes exhibited by the anurans, that a common methodology will be applicable across the anura. Therefore, further investigation into sperm cryopreservation protocols for anuran gene banking, through the use of research model species, and the application of successfully developed techniques to target species, must be pursued more vigorously. Recent studies on spermic urine cryopreservation,
specifically, are encouraging, with high post-thaw sperm motility and live young reported (Shishova et al., 2011; Uteshev et al., 2013).

The present paper reviews the techniques used and the results published in the literature and outlines the initial stages of development of a cryopreservation protocol using simple, inexpensive equipment. This paper offers a general approach, rather than a specific method, for cryopreservation of anuran sperm to ascertain the feasibility of developing a common sperm cryopreservation protocol through a relatively new technology - cryopreservation of hormonally-induced anuran sperm. The Fowler’s toad (A. fowleri) has provided a reliable model for preliminary investigation of sperm cryopreservation methods in the Bufonidae and target Bufonids for this study included the declining Southern Rocky Mountain boreal toad (Anaxyrus boreas boreas), and the critically endangered Puerto Rican crested toad (Peltophryne lemur).

The objectives of this study were to (1) evaluate the effect of exposure to two commonly used permeating cryoprotectants (DMSO and DMFA) on the membrane integrity and motility of spermatozoa; (2) compare the efficacy of the two cryoprotectants at different levels of concentration (5% and 10%) on the cryopreservation of spermatozoa from three Bufonid species; (3) evaluate the fertilization capacity and developmental outcomes of frozen-thawed A. b. boreas sperm through artificial fertilization. The overall objective was to ascertain a successful and reliable cryopreservation protocol for potential inter-species transfer across the Bufonidae, and to other threatened and endangered anuran species.
Materials and Methods

All animal procedures were conducted following review and approval by the Mississippi State University Institutional Animal Care and Use Committee.

Animals

Fowler’s toad (Mississippi State University)

Fowler’s toads (n =12) were housed in ventilated polycarbonate containers (30 L x 46 W x 66 H cm) with access to water and shelter. Frogs were maintained between 20–23°C and provided with standard fluorescent lights on a natural light cycle. Single-sex groups of 4-5 individuals were housed per container and offered a variety of food items including crickets, wax worms, and mealworms, three times per week. Crickets were gut-loaded with Repashy SuperLoad© supplement (Repashy Ventures Inc., CA, USA) and dusted with Reptivite © powder (ZooMed Laboratories, Inc., Costa Mesa, California, USA) prior to being fed out.

Puerto Rican crested toad (Omaha’s Henry Doorly Zoo)

Male PCR toads (n=4) were housed at Omaha’s Henry Doorly Zoo in same sex groups of 4-5 individuals in polycarbonate containers (30 cm L x 46 cm W x 66 cm H) on a natural light cycle with access to water and shelter. Toads were fed a variety of prey items each week, including crickets, mealworms and red wiggler worms.

Boreal toad (Native Aquatic Species Restoration Facility)

Male (n=7) and female (n=3) boreal toads housed at NASRF were maintained on a natural light cycle in large fiberglass tanks (121 x 60 x 30 cm) with a continuous supply of fresh water. Toads were fed a variety prey items three times per week, including
gutloaded (BugBurger, Allen’s Repashy, La Jolla, California, USA) crickets and mealworms, red-wiggler worms and waxworms. Crickets were dusted with Reptivite © powder (ZooMed Laboratories, Inc., Costa Mesa, California, USA) prior to feeding.

**Tadpoles**

On hatching (Gosner stage 20) (Gosner, 1960) all tadpoles were transferred from 150 mm petri dishes into larger plastic tubs (28 cm L x 20 cm W x 28 cm H) and fed ground tropical fish flakes (Tetra®, United Pet Group, Blacksburg, VA, USA) 1-2 times per day and algae pellets (Tetra®) *ad libitum*. Partial water changes (30 - 50%) were performed every 48 h. Upon emergence of front limbs (Gosner stage 42), tadpoles were removed into smaller tubs (50 cm L x 25 cm W x 35 cm H) containing 1 L aged tap water and monitored through metamorphosis.

**Spermic urine induction, collection and analysis**

Prior to hormone stimulation, a urine sample (T₀) was obtained from each male by holding the individual above a petri dish until urination occurred, usually within one minute evaluated for a spermiation response (determined by the presence or absence of sperm in the collected urine sample). Spermiation was induced by intraperitoneal injection of either 4 IU g⁻¹ body weight ([BW]; PCR toads) or 10 IU g⁻¹ BW (Boreal and Fowler’s toads) human chorionic gonadotropin ([hCG]; #C1063; Sigma Aldrich, St Louis, Missouri, USA).

Urine was collected from each male into sterile 1.5ml Eppendorf tubes at 2, 3, 5 and 7 h post-hormone administration (PA). The following spermatozoa quality parameters were evaluated: percentage spermatozoa Total Motility ([TM] = flagellum
movement); percentage Forward Progressive Motility ([FPM] = sperm with forward movement as a proportion of total sperm count), and FPM Quality ([FPM-Q]; 0 – 5 scale from no forward motility (0) to very rapid forward motility (5). Sperm motility parameters were analyzed immediately post-collection by placing a 10 µl aliquot of spermic urine under a 400x objective on a phase-contrast microscope (Olympus CX41) and counting the first 100 sperm. Sperm concentration was measured by inactivating sperm motility in a 1:10 dilution of phosphate-buffered saline (PBS) and counting on a Neubaeur haemocytometer to obtain average sperm concentration mL⁻¹.

**Study 1: CPA Cryotoxicity evaluation**

The toxicity of two penetrating Cryoprotective Agents (CPAs) was investigated using the Fowler’s toad (n=6) as a model species. Spermic urine samples were collected as per the methodology described above and freshly collected spermatozoa TM and viability were evaluated as described below.

**Cryoprotectant solutions**

Table 2 shows the two penetrating cryoprotectants investigated for toxicity effects comprised of a 0.5 M trehalose diluent (# T5251; Sigma-Aldrich, St. Louis, Missouri, USA) containing a final concentration of either 10% (v/v) dimethyl formamide ([DMFA] # D4551, Sigma-Aldrich) or 10% (v/v) dimethyl sulfoxide ([DMSO] # 47321, Sigma-Aldrich).
Table 2 The composition of four trehalose-based CPA solutions with different levels of penetrating cryoprotectant

<table>
<thead>
<tr>
<th></th>
<th>CPA A</th>
<th>CPA B</th>
<th>CPA C</th>
<th>CPA D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trehalose</td>
<td>0.5 M</td>
<td>0.5 M</td>
<td>0.5 M</td>
<td>0.5 M</td>
</tr>
<tr>
<td>DMSO</td>
<td>-</td>
<td>-</td>
<td>20%</td>
<td>10%</td>
</tr>
<tr>
<td>DMFA</td>
<td>20%</td>
<td>10%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stock osmolality (mOsm kg⁻¹)</td>
<td>896</td>
<td>496</td>
<td>4832</td>
<td>2072</td>
</tr>
<tr>
<td>Cryosuspension osmolality</td>
<td>504.9 ± 6.9</td>
<td>394.0 ± 7.1</td>
<td>1978.5 ± 32.9</td>
<td>1124.9 ± 13.8</td>
</tr>
</tbody>
</table>

Four cryoprotectant solutions comprised of a 0.5 M trehalose diluent containing either DMSO (10% or 20% (v/v) or DMFA (10% or 20% v/v) to give final concentrations of 5% and 10% DMSO or DMFA, on dilution in spermic urine.

Sperm motility and viability analysis urine

Cryoprotectant solutions were prepared at double strength of the final concentrations, followed by mixing with spermic urine (100µl) at a ratio of 1:1 (hereafter referred to as a cryosuspension) for toxicity analysis. Motility and viability were estimated prior to addition of CPA solution and after an equilibration period of 10, 20, 40 or 60 min. At each equilibration period, a 10 µl and a 5 µl aliquot of the cryosuspension were removed for sperm motility and viability analysis, respectively. Spermatozoa motility was analyzed by reactivating 10 µl of sperm suspension in a 1:10 dilution with aged tap water under a x400 objective on a phase contrast microscope (Olympus CX41). Spermatozoa membrane integrity was evaluated with the fluorescent dyes (SYBR)-14® and propidium iodide (PI) (live/dead viability kit; # L-7011; Invitrogen Scientific, Grand Island, NY, USA). A 5 µl aliquot of cryosuspension was stained 1:1 with a 1:50 dilution of the nucleic acid stain SYBR-14 for 10 min and 2 µl of PI for a further 10 min. A 10 µl aliquot was then placed on a glass slide and viability was evaluated under a 400x
objective on fluorescence microscope (Nikon Eclipse, Ni) at a wavelength of 490 nm. The first 100 spermatozoa were counted at random. Sperm stained with SYBR-14 alone were considered membrane-intact and sperm stained with both SYBR-14 and PI or PI alone were considered membrane-compromised (Figure 15).

Figure 15  *A. fowleri* spermatozoa stained with fluorescent dyes (SYBR)-14® and propidium iodide (PI) to ascertain membrane integrity

From left to right: Membrane-intact spermatozoa stained with SYBR-14; Membrane compromised spermatozoa stained with SYBR-14 and PI (middle) or PI alone (right).

**Study 2: Spermic urine cryopreservation and post-thaw motility**

Four cryoprotectant solutions were investigated using spermic urine collected from three toad species (*A. fowleri, A. b. boreas* and *P. lemur*) to determine the effectiveness of each CPA in protecting sperm cells during the cryopreservation process. Cryoprotectant solutions were prepared at double strength of the final concentration (Table 2).

Spermic urine samples were selected for cryopreservation if they met the following quality standards: >70% total motility, > 25% FPM with progressive motility quality ≥ 2 and sperm concentration > 0.8 x 10⁶ mL⁻¹. In order to guarantee 8-10 straw
replicates for each male, representing each CPA [A, B, C and D], spermic urine volumes of 3.2 mL – 4.0 mL were required (100µl cryosuspension/straw x 8-10 straws x 4 CPAs). It is rare to achieve these volumes from one urine collection so spermic urine samples from different collection time-points were often pooled together for each male. Spermatozoa motility parameters of pooled samples were analyzed immediately prior to cryopreservation so as to provide standardized spermic urine quality parameters for post-thaw evaluation of motility recovery. Cryoprotectant solutions were prepared at double strength of final concentrations (Table 2), prior to mixing with spermic urine aliquots (100 µl) at a ratio of 1:1. Cryosuspensions (200µL) were equilibrated at 4 °C for 10 min before loading into 0.25 mL plastic freezing straws [Minitube International®, Germany] (x 8 straws/CPA) and plugging with Critoseal® [Leica BioSystems, IL, USA]. Equilibration time incorporated the time of exposure to the cryoprotectant and the processing time from the addition of cryoprotectant until Step 1.

**Cryopreservation Step 1:** Removal of straws from 4° C and transfer to a
Styrofoam box (29 cm L x 2 cm W x 23 cm H) containing a platform situated 10 cm above a 3.5 cm depth of liquid nitrogen (Figure 16).

**Cryopreservation Step 2:** Straws held for 10 min in liquid nitrogen vapor before plunging directly into liquid nitrogen. Figure 17 details the freezing rate of this slow-rate method (Figure 17).

The thawing procedure involved immersion of a straw in a 40 °C water bath until all visible ice crystals had melted (~ 5 s). Thawed sperm contents were expelled into a small petri dish and further diluted with dechlorinated water at a ratio of 1:10 (1 part
cryosuspension: 1 part water) to activate spermatozoa motility. The following parameters were recorded 10 ± 5 sec after activation: Spermatozoa TM, FPM and FPM-Q.

Figure 16  Sperm cryopreservation equipment for slow-rate freezing

Sperm cryopreservation equipment: (A) 0.25 mL plastic freezing straws on loading tray; (B) Styrofoam box containing liquid nitrogen; (C) removable tray for the placement of freezing straws; (D) tray compartment secured 10 cm above the level of the liquid nitrogen; (E) removal of a false bottom allows the straws to drop from the tray directly into the liquid nitrogen below, without having to open the lid or interfere with the sample.
Cryosuspensions were maintained at 4°C for an equilibration period of 10 min and loaded into 0.25 mL freezing straws for a slow-cooling process of 20-25°C min⁻¹ over liquid nitrogen vapor. After 10 min straws were plunged into liquid nitrogen. Straws were thawed in a 40°C water bath for 5 s

**Fertilization capacity of chilled and frozen-thawed sperm**

**Ovulation and egg deposition**

Sexually mature female boreal toads (n=3) from a designated research colony at NASRF were held in individual plastic holding tubs (18 cm x 18 cm x 10 cm) with access to water, until oocyte deposition occurred. Stimulation of ovulation and oocyte deposition was achieved using the following exogenous hormone protocol: two hCG priming doses (3.7IU g⁻¹) four days apart, followed 24hr later by a combined ovulatory dose of hCG (3.7 IU g⁻¹) and luteinizing hormone releasing hormone analogue (0.4 µg g⁻¹) (GnRHa; [des-Gly¹⁰, D-Ala⁶]-LH-RH ethylamide acetate]cat# L4513; Sigma-Aldrich). On
initiation of egg deposition, females were gently squeezed to facilitate egg expression into 150mm petri dishes.

For fertilization trials using fresh spermatozoa, male boreal toads (control males; \( n=3 \)) were held in an individual plastic holding tub (18 cm x 18 cm x 10 cm) containing 2 cm water and administered 10 IU g\(^{-1}\) body weight (BW) hCG. Sperm was collected at 2 and 5 h post-hormone administration (PA) and analyzed as previously described. Frozen-thawed spermatozoa (\( n=6 \) straws/male) was cryopreserved as described above [Methods 5.2.3] using CPA A [Final concentration: 0.5 M trehalose (w/v) + 10% DMFA (v/v)].

Each female (\( n=3 \)) was represented by 6 fertilization dishes (3 x fresh sperm dishes; 3 x frozen sperm dishes). Fresh oocytes (75 ± 5 per dish) were deposited into 150 mm petri dishes and 100 µl sperm was pipetted onto the egg mass for a dry fertilization period of 5 min before flooding the dish with aged tap water. Parthenogenic control dishes (\( n=3 \)) were mixed with non-spermic urine to control for cleavage activated by means other than spermatozoa. Fertilization rates were assessed by counting the number of 2-4 cell embryos, 4-6 h post-fertilization. The percentage of tail-bud and tadpole stage embryos were calculated as a percentage of fertilized oocytes. On reaching tadpole stage (Gosner Stage 25), densities were standardized to 15 tadpoles in 3 replicates for both fresh and frozen-thawed derived tadpoles and monitored through metamorphosis. The percentage of metamorphosed tadpoles was calculated as a percentage of the total number of tadpoles per tub (\( n=15 \)).

**Statistical Analysis**

Assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk and Levene’s test, respectively. Percentage data were arcsine transformed
using the transformation $\sin^{-1}(\sqrt{x})$ before further analysis. For the cryotoxicity data, paired samples $t$-tests were used to analyze motility and viability recovery between fresh sperm samples and cryosuspensions (1:1 ratio of spermic urine mixed with CPA [DMSO or DMFA]) at each equilibration period (10, 20, 40, or 60 min). Spermatozoa motility and viability parameters were considered within each CPA at each equilibration period by a one-way ANOVA with cryoprotectant and equilibration time as fixed treatment variables and the dependent variables, sperm motility and viability (membrane intact). Independent samples $t$-tests were used to analyze differences between mean spermatozoa motility and viability of cryosuspensions equilibrated in each CPA, within an equilibration period.

All collected data on spermatozoa motility [TM and FPM] recovery following cryopreservation in each CPA (A, B, C and D) were considered in a one-way analysis of variance (ANOVA) with cryoprotectant as a fixed treatment variable and sperm motility parameters the dependent variable. The individual males were nested within treatment to remove variation among individual motility from the error term and the control (fresh sperm) was included in the model. Significant main effects were explored using Tukey-Kramer Honestly Significant Difference (HSD) post-hoc tests. Fertilization parameters under study were analyzed as means of the accumulated data from all replicates ($n=3$) of each male per treatment [Fresh or Frozen-thawed sperm] using paired samples $t$-tests. Values are expressed as means $\pm$ SEM and significance was established at $P \leq 0.05$. All statistical analysis was performed in SAS Version 9.4 (Cary, North Carolina, USA).
Results

Study 1: CPA cryotoxicity evaluation

Cryotoxicity trials were performed to ascertain the potential toxic effects of a trehalose-based diluent containing one of two different penetrating cryoprotectants (10% (v/v) DMSO or DMFA). Motility and viability recovery of spermatozoa from the model research species *A. fowleri* was estimated in this study. There was no significant difference between fresh sperm membrane integrity (88.2 ± 2.2%) and sperm membrane integrity recovered after a period of equilibration in DMSO or DMFA, for each equilibration duration (paired *t*-tests; *P*>0.05). However, there was a significant difference between fresh sperm motility (79.8 ± 3.4 %) and sperm motility following a period of equilibration in both DMSO and DMFA at all equilibration durations (paired *t*-tests; *P*<0.05). There was no significant difference in the relative recovery (recovered value/initial value x 100) of sperm motility between each equilibration time (10, 20, 40 and 60 min) within the DMSO-based treatment (one-way ANOVA; *F*$_{3,20}$=0.7; *P*=0.56) or within the DMFA-based cryoprotectant solution (one-way ANOVA; *F*$_{3, 20}$=0.64; *P*=0.59; Table 3). Similarly, there was no significant difference in the relative proportion of membrane-intact sperm between each equilibration time within the DMSO-based (one-way ANOVA; *F*$_{3, 20}$=0.51; *P*=0.68) or the DMFA-based cryoprotectant solution (one-way ANOVA; *F*$_{3, 20}$=0.29; *P*=0.83; Table 3). Further comparisons were made between sperm motility and membrane integrity between each cryoprotectant solution within an equilibration period using independent samples *t*-tests. There was no significant difference between CPA and equilibration period in relation to sperm motility and membrane integrity within an equilibration time (*P*>0.05; Table 3).
In summary, equilibration of *A. fowleri* sperm in a 0.5 M trehalose diluent comprising a final concentration of either 10% (v/v) DMSO or 10% (v/v) DMFA for up to 60 min did not compromise sperm membrane integrity. However, sperm motility was reduced following incubation at all time-points (10, 20, 40 and 60 min) in both cryoprotectants. Sperm viability, normalized as a proportion of fresh sperm viability, was high with ≥84% membrane intact sperm recovered across all incubation times for both CPAs. Sperm motility was reduced by up to 30%, relative to fresh sperm, on recovery from equilibration at all time-points in both CPAs, indicating that a proportion of sperm lost motility function over the course of the treatment but remained viable (Table 3).

### Table 3  Effect of exposure to a cryoprotectant (CPA) solution on *Anaxyrus fowleri* sperm motility (MOT) and viability (VIA) recovery

<table>
<thead>
<tr>
<th>CPA</th>
<th>10min</th>
<th>20min</th>
<th>40min</th>
<th>60min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MOT</td>
<td>VIA</td>
<td>MOT</td>
<td>VIA</td>
</tr>
<tr>
<td>DMSO</td>
<td>80.4 ± 6.0</td>
<td>89.7 ± 3.9</td>
<td>73.8 ± 13.4</td>
<td>86.5 ± 5.9</td>
</tr>
<tr>
<td>DMFA</td>
<td>72.5 ± 8.8</td>
<td>87.2 ± 5.9</td>
<td>72.5 ± 5.9</td>
<td>89 ± 3.6</td>
</tr>
<tr>
<td>8.8 ± 2.7</td>
<td>9.2 ± 3.7</td>
<td>9.2 ± 3.1</td>
<td>8.8 ± 5.3</td>
<td></td>
</tr>
</tbody>
</table>

Anaxyrus fowleri (n=6) sperm motility and membrane integrity recovery following a period of up to 60 min in a cryoprotectant (CPA) solution comprising either 0.5 M trehalose with final concentrations of either 10% (v/v) DMSO or 10% (v/v) DMFA. There was no significant difference (P>0.05) in spermatozoa viability or motility recovery between each incubation period (10, 20, 40, and 60 min) for both CPA treatments. Similarly, there was no significant difference (P>0.05) between sperm motility and viability parameters between each CPA at each incubation period. Relative motility values are post-thaw percentages of initial motility values. Means ± SEM.

**Study 2: Spermic urine cryopreservation and post-thaw motility**

Sperm cryopreservation trials were performed with the addition of two lower DMSO and DMFA levels (Final concentration: 5% and 10% [v/v]) to ascertain a
minimum effective concentration for sperm cryopreservation (Table 2). Males ($n=6$) from three Bufonid species ($A. fowleri$, $A. b. boreas$ and $P. lemur$) were utilized for this trial. The percentage of motility of spermatozoa was used as a parameter to evaluate viability. Overall, sperm motility parameters [TM and FPM] were affected by the cryopreservation process with significantly reduced post-thaw sperm motility and forward motility, relative to the corresponding fresh sperm sample, in all three species ($P<0.05$).

Frozen-thawed sperm from $A. fowleri$ exhibited significantly higher post-thaw sperm motility following cryopreservation in CPA A (10% DMFA) and CPA C (10% DMSO), with $69.1 \pm 1.7\%$ and $69.3 \pm 2.2\%$, respectively, compared to CPA D (5% DMSO), with $56.9 \pm 2.7\%$, respectively (Tukey-Kramer; $P<0.05$; Figure 18). However, there was no significant difference in the proportion of motile $A. fowleri$ sperm exhibiting forward progressive motility across all four cryoprotectants with $38.9 \pm 1.4\%$, $44.0 \pm 2.1\%$, $41.5 \pm 2.4\%$ and $35.4 \pm 2.1\%$ for cryoprotectant solutions A, B, C and D, respectively (Tukey-Kramer; $P>0.05$; Figure 18).
Figure 18  Effect of freeze-thawing on the motility parameters of sperm from *Anaxyrus fowleri* cryopreserved in four different cryoprotectant solutions

Sperm motility parameters [TM: white bar and FPM: grey bar] of fresh, and frozen-thawed sperm from (A) *A. fowleri* following cryopreservation in one of four 0.5 M trehalose-based cryoprotectant solutions comprising final concentrations of either: (A) 10% DMFA (v/v) (B) 5% DMFA (v/v) (C) 10% DMSO (v/v) (D) 5% DMSO (v/v).

Spermatozoa motility parameters were significantly reduced, relative to fresh sperm, after cryopreservation in all four CPA solutions. Significantly higher *A. fowleri* spermatozoa motility was recovered following cryopreservation in CPA A and CPA C, compared to CPA D. 

Values superscripted with the same letter do not differ significantly (*P* >0.05). *P* <0.05 compared with frozen-thawed. Data are expressed as untransformed means ±SEM.

Post-thaw sperm motility in *A. b. boreas* was highest in CPA A (10% DMFA) and CPA C (10% DMSO) with 48.9 ± 1.6% and 49.3 ± 2.2%, respectively. Cryoprotectants B and D produced slightly lower sperm motility with 42.7 ± 1.7% and 42.9 ± 1.8%, respectively. However, there was no significant difference in post-thaw sperm motility between each cryoprotectant (Tukey-Kramer; *P* >0.05; Figure 19.A). Similarly, there was no significant difference in the proportion of *A. b. boreas* sperm exhibiting forward movement between all four cryoprotectants with 23.2 ± 1.8%, 25.5 ± 1.7%, 27.5 ± 1.8%
and 23.2 ± 1.3% for cryoprotectants A, B, C and D, respectively (Tukey-Kramer; $P>0.05$).

The highest post-thaw sperm motility from *P. lemur* was from sperm frozen in CPA A (50.6 ± 2.0%), however this did not differ significantly from post-thaw sperm motility following cryopreservation in CPA B, C or D with 47.2 ± 1.6%, 45.2 ± 2.4% and 43.6 ± 2.3%, respectively (Tukey-Kramer; $P>0.05$; Figure 19.B). Consistent with *A. fowleri* and *A. b. boreas* the proportion of post-thaw *P. lemur* sperm exhibiting forward motility was not significantly different across all CPAs, with 37.7 ± 2.4%, 33.5 ± 2.2%, 28.2 ± 3.2% and 29.5 ± 2.4%, following cryopreservation in CPA A, B, C and D, respectively (Tukey-Kramer; $P>0.05$; Figure 19.B).
Figure 19  Effect of freeze-thawing on the motility parameters of sperm from *Anaxyrus b. boreas* and *Peltophryne lemur* cryopreserved in four different cryoprotectant solutions

Sperm motility parameters [TM: white bar and FPM: grey bar] of fresh, and frozen-thawed sperm from (A) *A. b. boreas* and (B) *P. lemur* following cryopreservation in one of four 0.5 M trehalose-based cryoprotectant solutions comprising final concentrations of either: (A) 10% DMFA (v/v) (B) 5% DMFA (v/v) (C) 10% DMSO (v/v) (D) 5% DMSO (v/v). Spermatozoa motility parameters were significantly reduced, relative to fresh sperm, after cryopreservation in all four CPA solutions for both *A. b. boreas* and *P. lemur* and there was no significant difference in recovered spermatozoa motility parameters between cryoprotectant solutions for either species. \(^{ab}\)Values superscripted with the same letter do not differ significantly \((P>0.05)\). \(^*\)\(^{P}<0.05\) compared with frozen-thawed. Data are expressed as untransformed means ±SEM.
A. fowleri sperm quality following cryopreservation in CPA A and CPA C was not significantly reduced, compared to fresh sperm with 4.5 ± 0.1 and 4.8 ± 0.1, respectively (Tukey-Kramer; *P* > 0.05; Figure 20). Within the CPA treatments, A. fowleri post-thaw sperm quality was significantly higher following cryopreservation in CPA C (4.9 ± 0.1) compared to CPA B (4.06 ± 0.2) and CPA D (4.1 ± 0.2), but did not significantly differ from sperm quality recovered from cryopreservation in CPA C (4.6 ± 0.1).

Post-thaw sperm quality was significantly lower in all four CPAs, relative to fresh sperm quality in A. b. boreas and P. lemur (Tukey-Kramer; *P* < 0.05; Figure 20). Sperm quality of A. b. boreas did not significantly differ following cryopreservation in all four cryoprotectant solutions, ranging from 3.3 ± 0.2 - 3.9 ± 0.2 (Tukey-Kramer; *P* > 0.05; Figure 19). P. lemur sperm cryopreserved in CPA A (3.5 ± 0.3) and CPA B (3.7 ± 0.3) exhibited significantly higher post-thaw sperm quality than sperm frozen using CPA C (2.6 ± 0.3) and CPA D (2.7 ± 0.3; Tukey-Kramer; *P* < 0.05; Figure 20).
Figure 20  Effect of freeze-thawing on the quality of forward movement of *Anaxyrus. Fowleri, A. b. boreas*, and *Peltophryne lemur* sperm cryopreserved in four different cryoprotectant solutions

The quality of post-thaw sperm forward movement [FPM-Q] from fresh and frozen-thawed *A. fowleri* [black bar], *A. b. boreas* [grey bar], and *P. lemur* [white bar] sperm following cryopreservation in one of four 0.5 M trehalose-based freezing extenders comprising a final concentration of either: (A) 10% DMFA (v/v) (B) 5% DMFA (v/v) (C) 10% DMSO (v/v) (D) 5% DMSO (v/v). There was a significant effect of the cryopreservation process on the post-thaw sperm quality of *A. b. boreas* and *P. lemur* sperm with fresh sperm quality significantly higher than frozen-thawed sperm quality (*P*<0.05). Post-thaw sperm quality of *A. fowleri* sperm cryopreserved in CPA A or CPA B did not differ significantly from fresh sperm quality (*P*>0.05), however the quality of frozen-thawed sperm from CPA B and CPA D was significantly reduced following cryopreservation (*P*<0.05). There was no significant difference in post-thaw *A. b. boreas* sperm quality recovered following cryopreservation in all four CPAs (*P*>0.05). *P. lemur* sperm recovered from CPA A and CPA B was of a significantly higher quality than that of sperm frozen in CPA C or CPA D. abcValues superscripted with the same letter within a species are not significantly different (*P*>0.05). Values are expressed as untransformed means ±SEM.

In summary, there was a significant reduction in *A. fowleri, A. b. boreas* and *P. lemur* sperm motility parameters, relative to fresh sperm, following cryopreservation in each CPA (A, B, C and D). There was no significant difference in frozen-thawed sperm motility [TM and FPM] between all four cryoprotectant solutions, in *P. lemur* and *A. b. boreas*, however *A. fowleri* sperm exhibited significantly higher post-thaw motility in
CPA A (10% DMFA) and CPA C (10% DMSO), compared to CPA D (5% DMSO). The proportion of motile sperm exhibiting forward movement was not significantly different across all four CPAs for all three species. The quality of forward motile sperm did not significantly differ across all four CPAs in *A. b. boreas*, however *A. fowleri* sperm cryopreserved in CPA C was of a significantly higher quality than frozen-thawed sperm from CPA B or CPA D. Post-thaw sperm quality of *P. lemur* sperm frozen in CPA A and CPA B was of a significantly higher quality than sperm frozen in CPA C or CPA D (Figures 18, 19 and 20).

**Study 3: Fertilization capacity of frozen-thawed sperm: *A. b. boreas***

Cryopreservation trials indicated that there was no significant difference in the post-thaw motility recovery of *A. b. boreas* sperm following cryopreservation in all four CPAs (Figure 19). Cryoprotectant solution A (0.5 M trehalose + 10% DMFA) was selected for artificial fertilization (AF). Post-thaw *A. b. boreas* (*n*=3) sperm motility parameters [TM and FPM] were significantly reduced, relative to fresh sperm, following cryopreservation in CPA A (Paired *t*-tests; *P*<0.05; Figure 21.AandB.).

Frozen-thawed *A. b. boreas* motility was 56.8 ± 1.4%, 50.5 ± 3.1% and 67.8 ± 3.4%, for males 1, 2 and 3, respectively. If data are normalized to the pre-freeze value, this equates to a relative recovery of 62%, 65% and 73% motility of the fresh (control) sperm sample, for males 1, 2 and 3, respectively (Figure 21. A.). Post-thaw sperm forward motility was 30.2 ± 3.0%, 31.3 ± 3.6% and 44.0± 3.6%, with normalized values of 43%, 59% and 59%, for males 1, 2 and 3, respectively (Figure 21.B.). Fresh sperm quality was ranked a 5 for all males and post-thaw quality was significantly lower with 3.3 ± 0.2, 4.0 ± 0.2 and 4.0 ± 0.3 for males 1, 2 and 3, respectively (*P*<0.05).
Overall, fertilization rates from AF trials were relatively low and the fertilization capacity of fresh and frozen-thawed *A. b. boreas* sperm did not significantly differ (P>0.05), with 32.2 ± 12.9% and 17.6 ± 5.7%, respectively (Table 4). The proportion of embryos reaching neurula and tadpole stage did not significantly differ between embryos fertilized with either fresh or frozen-thawed *A. b. boreas* sperm (P>0.05; Table 4). Specifically, of the fertilized embryos 84.3 ± 9.5% and 74.9 ± 12.3% reached neurula stage, with the majority (79.0 ± 12.2% and 72.3 ± 12.7%) continuing development to tadpoles, from fresh sperm and frozen-thawed sperm AF trials, respectively (Table 4). Parthenogenic control dishes exhibited 7.3 ± 2.7% of embryos with poor-quality cell division, most likely mechanically activated during egg retrieval. The percentage of fully-metamorphosed tadpoles derived from fresh (37.8 ± 18.1%) and frozen-thawed (51.0 ± 5.9%) sperm artificial fertilizations did not differ significantly (t=0.7; P=0.52; Figure 22).

Overall, post-thaw sperm motility parameters were significantly lower than the corresponding fresh sperm samples for all three male *A. b. boreas*. However, there was no significant effect of the cryopreservation process on the developmental outcomes of artificial fertilization using frozen-thawed *A. b. boreas* sperm.
Figure 21  Effect of freeze-thawing on *Anaxyrus b. boreas* sperm motility parameters

The percent (A) Total Motility (TM) and (B) Forward Progressive Motility (FPM) of fresh [white bar] and frozen-thawed [spotted bar] *A. b. boreas* (n=3 males; n=6 straws per male) sperm following cryopreservation in a 0.5 M trehalose diluent comprising a final concentration of 10% DMFA. Post-thaw sperm motility parameters were significantly lower than fresh sperm in all three males (P<0.05). Values are expressed as untransformed means ± SEM
Table 4  Effect of sperm exposure to freeze-thawing on the developmental outcomes of *Anaxyrus b. boreas* embryos and tadpoles reared at 20° C

<table>
<thead>
<tr>
<th></th>
<th>No. eggs</th>
<th>Cleavage (%)</th>
<th>Neurula (%)</th>
<th>Tadpole (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh sperm</td>
<td>739</td>
<td>32.2 ± 12.9</td>
<td>84.3 ± 9.5</td>
<td>79.0 ± 12.2</td>
</tr>
<tr>
<td>Frozen-thawed sperm</td>
<td>584</td>
<td>17.6 ± 5.7</td>
<td>74.9 ± 12.3</td>
<td>72.3 ± 12.7</td>
</tr>
<tr>
<td>Parthenogenic control</td>
<td>183</td>
<td>7.3 ± 2.7</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The developmental stages of embryos produced from artificial fertilization trials using fresh and frozen-thawed *A. b. boreas* sperm. There was no significant effect of the cryopreservation process (*P* > 0.05) on the proportion of embryos at each developmental stage. Values are expressed as means of the total number of eggs laid (cleavage) or the total number of fertilized eggs (neurula and tadpole) ± SEM.

Figure 22  Effect of freeze-thawing on the percentage of *A. b. boreas* tadpoles

The percentage of metamorphosed tadpoles produced from artificial fertilization (AF) trials using fresh and frozen-thawed *A. b. boreas* (*n*=3) sperm. There was no significant effect of the cryopreservation process on the average number of tadpoles completing metamorphosis from all three male AF trials (*P* > 0.05). Values are expressed as untransformed means of the total number of fertilized eggs ± SEM.
Discussion

Common species such as *A. fowleri* are valuable model systems in which to develop and refine cryopreservation protocols. In the present study fresh *A. fowleri* spermatozoa motility and membrane integrity were analyzed following incubation in cryoprotectant solutions (CPAs) to determine any potential effects of toxicity. Spermic urine samples from three different Bufonid species: *Anaxyrus fowleri*, *A. b. boreas* and *Peltophryne lemur*, were cryopreserved in each of four different CPAs, and sperm motility and fertilization capacity (*A. b. boreas* only) were used as assays of post-thaw sperm competence.

Although cryopreservation methods for freezing spermic urine have been applied to three species: *R. pipiens* (Hopkins and Herr, 2007), *Rana temporaria* (Shishova et al., 2011), and *Pelophylax lessonae* (Uteshev et al., 2013), a number of drawbacks could potentially limit the widespread application of this technique across the anurans. Chiefly, the ability to non-invasively obtain gametes could be a rate-limiting step for some species. Stimulation of sperm release into urine is generally achieved through exogenous hormone administration. Therefore, the development of a reliable and effective hormone regimen must precede cryopreservation. In the present study, hormone regimens for spermiation induction were previously developed on the Fowler’s toad (Kouba et al., 2009b) and subsequently refined and successfully transferred to *A. b. boreas* (Kouba et al., 2009b; Langhorne et al., unpublished) and *P. lemur* (Langhorne et al., unpublished). However, response to hormone treatments can be species-specific, therefore investigation of hormone regimens for gamete induction is a fundamental first-step in the successful generation of a gamete repository for endangered anuran species.
Furthermore, sperm concentrations obtained from urine samples can be orders of magnitude below those of testes macerates (Kouba et al., 2013). This could be problematic considering the mechanism of sperm motility activation, which requires exposure to a hypotonic environment (Fitzsimmons et al., 2007). The use of high osmolality CPAs results in substantial dilutions to attain osmolalities within the motility range of anuran sperm and this can result in oligozoospermic samples, which may affect fertilization rates. Consequently, spermic urine freezing may only be appropriate for sperm samples of relatively high volumes and concentrations and may not be suitable for the cryopreservation of sperm from small anuran species that do not release large volumes or concentrations of spermic urine.

Conventional cryopreservation (i.e. slow-rate freezing) has been applied to sperm of several common anuran species from a number of Genera, including Bufo, Xenopus, and Rana. A general review of the literature shows that fertilization has been achieved using frozen-thawed testicular sperm or spermic urine cryopreserved in CPAs comprising ionic or non-ionic diluents with permeating CPAs including DMSO, glycerol and DMFA of varying concentrations. However, standardized methodologies for the expanded application of anuran cryopreservation techniques is complicated by the wide literature base reporting a variety of different extender and freezing solutions, sperm packaging materials, and cooling and thawing rates (Table 5 and 6).
<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>CPA</th>
<th>Extender</th>
<th>Summary of findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bufo americanus</td>
<td>TM</td>
<td>EG (15%)</td>
<td>Alsever’s media</td>
<td>MOT 88%</td>
<td>Barton and Guttman 1972</td>
</tr>
<tr>
<td>Rana temporaria</td>
<td>TM</td>
<td>DMSO (15%)</td>
<td>0.25% NaCl + HEPES</td>
<td>No data available</td>
<td>Kaurova et al. 1996</td>
</tr>
<tr>
<td>Bufo marinus</td>
<td>TM</td>
<td>DMSO (15%) GLY (20%)</td>
<td>SC diluent</td>
<td>MOT 58 - 68%; FERT 60 - 81%</td>
<td>Browne et al. 1998</td>
</tr>
<tr>
<td>Rana sylvatica</td>
<td>TM</td>
<td>GLY, DMSO (1.5 M, 3.0 M)</td>
<td>SB</td>
<td>VIA ~10-20%</td>
<td>Mugnano et al. 1998</td>
</tr>
<tr>
<td>Rana pipiens</td>
<td>TM</td>
<td>GLY (0.5 M)</td>
<td>SB</td>
<td>VIA 48% - 81%</td>
<td>Beesley et al. 1998</td>
</tr>
<tr>
<td>Eleutherodactylus coqui</td>
<td>TM</td>
<td>FBS + DMSO (2 M) GLY, SC (2 M)</td>
<td>SDB</td>
<td>MOT 50 - 54%</td>
<td>Michael and Jones, 2004</td>
</tr>
<tr>
<td>Xenopus laevis and X. tropicalis</td>
<td>TM</td>
<td>EY (20%), SC (0.8 M) SBi (20 mM)</td>
<td>Leibovitz-L-15</td>
<td>MOT 10 - 40%; FERT 23 -77%</td>
<td>Sargent and Mohun 2005</td>
</tr>
<tr>
<td>Rana pipiens</td>
<td>SU</td>
<td>GLY (5.9%) SC (6.6%)</td>
<td>Salamon’s media</td>
<td>VIA 35%</td>
<td>Hopkins and Herr 2007</td>
</tr>
<tr>
<td>X. laevis</td>
<td>TM</td>
<td>DMSO (5%) SC (73 mM)</td>
<td>MIS</td>
<td>MOT 80%, HATCH 70%</td>
<td>Mansour et al. 2009</td>
</tr>
<tr>
<td>Rana temporaria</td>
<td>TM</td>
<td>GLY (5%) SC (2.5%) EY (5%)</td>
<td>MIS + 300 mM SC</td>
<td>VIA 45%, MOT 35%</td>
<td>Mansour et al. 2010</td>
</tr>
<tr>
<td>Rana temporaria</td>
<td>SU</td>
<td>DMFA (12%) SC (10%)</td>
<td>SAR</td>
<td>MOT 65%, VIA 72%, FERT 91%</td>
<td>Shishova et al. 2011</td>
</tr>
<tr>
<td>Pelophylax lessonae</td>
<td>SU</td>
<td>DMFA (12%) SC (10%)</td>
<td>SAR</td>
<td>MOT 40%, FERT 29%</td>
<td>Uteshev et al. 2013</td>
</tr>
</tbody>
</table>

Source: TM=Testes macerates; SU=Spermic urine. MOT=motility; VIA=viability; FERT=fertilization; HATCH=hatching
Cryoprotectants: Dimethyl formamide (DMFA); Dimethyl sulfoxide (DMSO); Egg Yolk (EY); Ethylene glycol (EG) Fetal bovine serum (FBS); Sodium Bicarbonate (SBI); Sucrose (SC). Extender: SB=suspension buffer (in mM: 104.4 NaCl, 2.0 KCl, 6.1 Na₂HPO₄, 1.0 KH₂PO₄ [230mOsm kg⁻¹, pH 7.4]); SDB=(10 mM NaCl, 0.2 mM KCl, 0.1 mM CaCl₂, 0.1 mM MgCl₂ and 0.5 mM Heps [pH7.5]); L-15 (0.15 M NaCl); MIS=motility-inhibiting solution (150 mM NaCl, 3.0 mM KCl, 1 mM Mg₂SO₄, 1.0 mM CaCl₂, and 20 mM Tris [pH8]); SAR=simplified amphibian ringers (In 1 Litre: 6.6 g NaCl; 0.15 g KCl, 0.15 g CaCl₂, 0.2 g NaHCO₃ [200-250 mOsm kg⁻¹]).
A review of slow cooling and thawing rates of cryopreserved sperm from a number of different anuran species

<table>
<thead>
<tr>
<th>Species</th>
<th>Packaging</th>
<th>Freezing rate ( ^\circ C \text{ min}^{-1} )</th>
<th>Thaw</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bufo americanus</em></td>
<td>Tube (SNS)</td>
<td>0.7</td>
<td>RT WB ( (25^\circ C) )</td>
<td>Barton and Guttman, 1972</td>
</tr>
<tr>
<td><em>Bufo marinus</em></td>
<td>0.25 mL straw</td>
<td>10</td>
<td>RT</td>
<td>Browne et al. 1998</td>
</tr>
<tr>
<td><em>Rana sylvatica</em></td>
<td>0.5 mL Eppendorf</td>
<td>130</td>
<td>RT (warm water)</td>
<td>Beesley et al. 1998</td>
</tr>
<tr>
<td><em>Rana sylvatica</em></td>
<td>Tube (SNS)</td>
<td>130</td>
<td>RT WB ( (30^\circ C) )</td>
<td>Mugnano et al. 1998</td>
</tr>
<tr>
<td><em>Eleutherodactylus coqui</em></td>
<td>Eppendorf (SNS)</td>
<td>25</td>
<td>RT WB ( (20^\circ C) )</td>
<td>Michael and Jones, 2004</td>
</tr>
<tr>
<td><em>Xenopus laevis</em> and <em>Xenopus tropicalis</em></td>
<td>0.25 mL straw and 0.5 mL Eppendorf</td>
<td>10</td>
<td>RT WB ( (30^\circ C) )</td>
<td>Sargent and Mohun, 2005</td>
</tr>
<tr>
<td><em>Bufo marinus</em></td>
<td>Tube (SNS)</td>
<td>3</td>
<td>RT (3 min)</td>
<td>Fitzsimmons et al. 2007</td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>50 µl mini-straw</td>
<td>20-25</td>
<td>RT ( (40 \text{ s}) )</td>
<td>Mansour et al. 2009</td>
</tr>
<tr>
<td><em>Rana temporaria</em></td>
<td>50 µl mini-straw</td>
<td>10</td>
<td>RT WB ( (22^\circ C) )</td>
<td>Mansour et al. 2010</td>
</tr>
<tr>
<td><em>Rana temporaria</em></td>
<td>0.25 mL straw</td>
<td>5-7</td>
<td>RT WB ( (40^\circ C) )</td>
<td>Shishova et al. 2011</td>
</tr>
<tr>
<td><em>Pelophylax lessonae</em></td>
<td>0.25 mL straw</td>
<td>5-7</td>
<td>RT WB ( (40^\circ C) )</td>
<td>Uteshev et al. 2013</td>
</tr>
</tbody>
</table>

RT=Rapid thaw; WB=Water bath SNS=size not specified
Development of a cryopreservation protocol

The successful development of a cryopreservation protocol must consider both the extender and cryopreservation solutions, as well as the cooling and thawing rates; the effects of which can all interact to influence the overall outcome of the cryopreservation procedure (Suquet et al., 2000).

Selection of sperm inactivation extender solution

Anuran spermatozoa are immotile in the testis and in solutions with osmolalities equal to or greater than blood plasma ≥200 mOsm kg\(^{-1}\) (Fitzsimmons et al., 2011). Since successful fertilization requires the sperm to reach, bind and penetrate the egg, the process of sperm motility activation is an essential pre-requisite (O’Brien et al., 2011). Initiation of sperm motility in the anurans requires exposure of the sperm to a hypotonic environment, such as spawning into water (Fitzsimmons et al., 2007; O’Brien et al., 2011). Freshly-collected anuran spermic urine is typically in the range of 20 – 80 mOSm kg\(^{-1}\) [pers. observ.], therefore, unlike testicular sperm from testis macerates, spermatozoa in urine are in an active state immediately on collection.

A diluent extender is a physiological solution that inactivates sperm, to which cryoprotectants can be added (Zhang and Lubzens et al., 2009). During cryopreservation, sperm is maintained in a quiescent state by exposure to hypertonic solutions of osmolalities greater than or equal to blood plasma (Fitzsimmons et al., 2007; O’Brien et al., 2011). In addition to selection of an effective CPA, selection of an extender is a key factor in the success of cryopreservation (Gao et al., 2000). The majority of anuran sperm cryopreservation protocols use ionic sperm extenders (Table 6). However, Browne et al.
(2002a) reported higher cryopreservation success using a sucrose rather than the saline-based solution which appeared to exert a deleterious effect on *B. marinus* sperm. In a separate study, also by Browne *et al.* (2002b) the effects of a number of saccharide diluents on the post-thaw recovery of *B. marinus* spermatozoa were compared. Disaccharides (e.g. sucrose, trehalose) were found to be more effective than monosaccharides (e.g. glucose, fructose) in the protection of sperm cells during slow cooling (Browne *et al.*, 2002b). Trehalose, in particular, has been shown in a number of studies to have a protective effect on cell membranes (Crowe, *et al.*, 1987, 1994; Storey *et al.*, 1998). Based on these data and preliminary cryopreservation trials in which comparisons of saline-based and sugar-based diluents did not appear to show differences in frozen-thawed recovery of *A. fowleri* sperm motility parameters [pers. observ.], trehalose was selected as the diluent base for the present study.

**Evaluation and selection of cryoprotectant**

Glycerol is one of the most effective cryoprotectants, particularly regarding sperm cryopreservation, however, in some species it can also be highly toxic (Watson and Fuller, 2001). Glycerol has been used effectively in the cryopreservation of sperm from *Bufo marinus* (Browne *et al.*, 1998) and *Rana sylvatica, R. pipiens* and *Bufo americanus* (Beesley *et al.*, 1998; Hopkins and Herr, 2007), *Rana temporaria* (Mansour *et al.*, 2010) and *Eleutherodactylus coqui* (Michael and Jones, 2004). Preliminary CPA trials, preceding the present study, showed poor post-thaw sperm motility recovery following incubation and cryopreservation in glycerol and was therefore excluded from the experiment.
Notionally, higher concentrations of cryoprotectant afford better protection to sperm cells during the freezing process (Yang et al., 2009). However, if concentrations are too high then they can become toxic or lethal to sperm, therefore a balance between these two effects is desired (Yang et al., 2009). Species-specificity can be an issue regarding susceptibility to CPA toxicity, and one of the primary steps in the development of a sperm cryopreservation protocol is selection of the least toxic but most effective cryoprotectant. The permeability of dimethyl sulfoxide (DMSO) is not markedly affected by low temperatures (Whittingham, 1977), which may be one reason why it has been widely adopted as a CPA across many taxa (Watson and Fuller, 2001). DMSO is characterized by a small molecular weight and a fast cellular penetration rate; however DMSO can also be toxic in high concentrations (Ogier de Baulny et al., 1996; Zhang et al., 2003). In anuran sperm cryopreservation, DMSO has successfully been used in concentrations of 5% - 20% (Beesley et al., 1998; Browne et al., 1998, 2002; Kaurova et al. 1996; Michael and Jones, 2004; Mansour et al., 2009ab). However, sensitivity exists even within this range. A concentration of 15% DMSO was shown to exert toxic effects on the sperm of Xenopus laevis (Buchholz et al., 2004), and reduced post-thaw sperm motility and larval hatching rates at concentrations of 10% (Mansour et al., 2009b). However, no significant effects on X. laevis post-thaw sperm recovery were observed at a lower concentration of 5% DMSO (Mansour et al., 2009b).

The novel cryoprotectant N, N-dimethylformamide (DMFA) is a more recent addition, having been successfully applied to spermic urine cryopreservation in two common model species with high rates of post-thaw sperm motility and fertility, and larval survival at final concentrations of 12% (Shishova et al., 2011; Uteshev et al., 2011).
2013). Shishova et al. (2011) reported the first use of a DMFA-based CPA for the cryopreservation of *Rana temporaria* spermic urine, extended in a sucrose diluent comprising 12% DMFA. Post-thaw sperm motility, viability and fertility recovered from cryopreservation in DMFA was superior to DMSO-based CPAs of the same concentration, although larval survival did not differ significantly (Shishova et al., 2011). Subsequently, Uteshev et al. (2013) applied the same freezing technique to the common pool frog (*Pelophylax lessonae*) and achieved 71% post-thaw membrane integrity, 40% post-thaw motility, and 29% fertilization rates.

In the present study DMSO and DMFA at final concentrations of 5% and 10% were selected for cryopreservation trials.

**Toxicity evaluation of cryoprotectant**

The majority of previous anuran sperm cryopreservation studies do not report a CPA toxicity trial or the motility parameters of fresh sperm prior to the freezing process. Therefore, extrapolation of the exact cause of any resulting post-thaw reduced function is difficult to discern; specifically, whether or not reduced capacity of frozen-thawed sperm resulted from the effects of CPA toxicity before freezing, or from cryoinjury during the freezing or thawing process. DMFA and DMSO selected for the current study were subject to toxicity testing prior to sperm cryopreservation using *Anaxyrus fowleri* as a model species. Sperm function was evaluated through viability staining techniques to ascertain membrane integrity, and sperm motility analysis. Sperm cell staining is a useful indicator of the overall viability of a sample, which may not necessarily be reflective of sperm motility. Incubation of *A. fowleri* sperm with the permeating cryoprotectants DMFA or DMSO (at a final concentration of 10%) indicated that there was no significant
difference between both CPAs in their effects on sperm membrane integrity.

Furthermore, there was no significant difference in sperm membrane integrity between fresh sperm and sperm incubated in DMSO or DMFA. Although, a period of incubation in DMFA or DMSO did significantly reduce sperm motility upon reactivation, relative to fresh sperm controls, for both cryoprotectants.

Due to the relatively small size of spermatozoa, intracellular penetration of cryoprotectant is rapid and a period of CPA equilibration may not necessarily be required (Jamieson, 1991). Observations by Ogier de Baulny (1996) showed that 10 minutes was required for DMSO to permeate rainbow trout (Oncorhynchus mykiss) spermatozoa, although post-thaw fertilization capacity did not differ between equilibrated and non-equilibrated spermatozoa. In contrast, the fertilization capacity of spermatozoa from yellowfin sea bream (Acanthopagrus latus) decreased following an equilibration duration in DMSO that exceeded just two minutes (Gwo, 1994). These observations indicate that a long period of equilibration may not be required for cell permeation by a cryoprotectant (Ogier de Baulny, 1996). As no significant difference between any of the four equilibration durations was observed in the present study, a 10 min CPA incubation was selected, based on the time taken to process and package one batch ($n=10$ straws) of sperm samples prior to freezing step one. Temperature plays an important role in exposure to cryoprotectants because it interacts with factors such as chilling sensitivity of the gametes, toxic properties of the cryoprotectant, and permeation rate (lower temperatures require a longer period of equilibration) (Cuevas-Uribe et al., 2011). In the present study spermic urine was incubated in CPA at 4° C due to the tendency of sperm motility to decrease with increasing time exposed to room temperatures [pers. observ.].
However, follow-up trials using a higher (10° C) and lower (0° C) exposure temperature would be worthwhile.

**Freezing and thawing rates**

Determination of the optimum freezing rate can further limit cryopreservation protocol development, and tolerance to freezing can be highly dependent on species, as reported among fish (Suquet *et al.*, 2000). Generally, there is an inverse relationship between freezing and thawing rate, whereby the more rapid the rate of freezing, the slower the rate of thawing (Mazur *et al.*, 2008). A variety of cooling and thawing rates have been reported in the literature (Table 6) but typically, the use of a two-step cooling procedure is applied. Specifically, sperm samples are cooled in liquid nitrogen vapor before immersion in liquid nitrogen, the cooling rate, therefore, is determined by the height of the samples above the level of liquid nitrogen. In the present study a cooling rate of 20 to 25° C min\(^{-1}\) was achieved by holding samples on a platform 10 cm above the level of liquid nitrogen, as reported by Mansour *et al.* (2010), Shishova *et al.* (2011) and Uteshev *et al.* (2013) (Figure 17).

Generally, thawing procedures applied subsequent to slow-rate freezing conditions involve immersion in a warm water bath (between ~ 20 - 40° C), or thawing at room temperature, depending on the type of sperm packaging (Table 6). The present study utilized 0.25 mL freezing straws containing a 200 µl cryosuspension, which took approximately 5 seconds for ice crystals to melt in a 40° C water bath. Room temperature thawing, where a straw removed from liquid nitrogen storage was thawed on the benchtop until ice crystals had melted (approximately 2.5 minutes) produced similar, if
slightly lower post-thaw motility [pers. observ.], therefore the 40° C water bath thawing technique was selected for this study.

**Post-thaw sperm motility and fertility recovery**

In external spawners there is generally a positive relationship between motility and fertility, and as such, motility is often used as an indicator of fertility in artificial fertilization due to the ease of assessment of motility (Gwo, *et al.*, 2009).

**Spermatozoa motility**

Following cryopreservation in four different CPA solutions (A, B, C and D), the model species Fowler’s toad recovered high rates (69%) of post-thaw sperm motility and quality using DMFA-based CPA A and DMSO-based CPA C, indicating that the higher concentration of cryoprotectant conferred better protection from the freezing process. In contrast, although Shishova *et al.* (2011) showed similar rates of post-thaw recovery for *R. temporaria* sperm frozen in 12% DMFA (65.3 ± 3.3%), post-thaw sperm motility recovery following cryopreservation in DMSO at the same concentration was significantly lower (43 ± 3.7%). Notably, in the present study, *A. fowleri* sperm quality recovered following cryopreservation in CPAs A and C did not differ from fresh sperm, despite a significant reduction in the overall proportion of motile spermatozoa. There was moderate overall post-thaw motility recovery of *A. b. boreas* and *P. lemur* spermatozoa following cryopreservation in each CPA but no significant difference in post-thaw results between all four CPAs. Sperm motility was significantly reduced from fresh sperm samples with motility recovery rates in the range of 42% -51% across all four CPAs. These results are comparable with those reported by Uteshev *et al.* (2013) using a
DMFA-based CPA at a concentration of 12%. In the present study, the sperm motility parameter of forward progression [FPM] was significantly reduced following the cryopreservation process in all four CPAs for all three species (between 23.2 ± 1.3% - 44.0 ± 2.1%). However, post-thaw FPM values were higher than those reported by Uteshev et al. (2013) with 10.0 ± 3.0% FPM in frozen-thawed *P. lessonae* spermatozoa.

Visual estimation of sperm motility does not always prove to be an accurate indicator of fertilization ability however, and among fish, immotile sperm have been shown to produce high fertilization rates (Gwo et al., 2009). For example, thawed striped bass (*Marone saxatilis*) spermatozoa achieved a fertilization rate of 88% (Gwo et al., 2009). On the contrary, sperm samples exhibiting high motility can produce poor fertilization rates [pers. observ.], which might be explained by poor egg quality, however another factor to consider is premature activation of the acrosome reaction. DMSO has been reported to prematurely trigger the acrosome reaction prior to contact with eggs in frozen-thawed sperm of the sturgeon (*Acipenser ruthenus*) frozen in DMSO, however sperm motility parameters were unaffected (Psenicka et al., 2008). Generally speaking, poor motility is a sign of decreased fertility, however motility remains the principal laboratory measure available for evaluating anuran sperm quality.

**Spermatozoa fertility**

Although motility is the most widely used assay for post-thaw sperm function, fertilization and the production of offspring are considered to be the most informative assessment for cryopreserved sperm (Watson and Fuller, 2001). The relatively simplistic nature of reproduction in the majority of anuran species makes techniques such as artificial fertilization (AF) reasonably straightforward to apply (Kouba et al., 2009).
Following hormone stimulation, gametes, expressed from males and females, can be mixed together in a dish for fertilization and the resulting embryos left to develop through metamorphosis with minimal input (Kouba et al., 2009). It would be impractical to induce eggs for ‘test-thawing’ the fertilization capacity of batches of cryopreserved sperm, particularly for species that may only spawn once a year, or in species where ovulation induction protocols have not yet been developed. Therefore, motility and membrane integrity assays are likely to be the principal quality control measures for anuran sperm banking. Optimal gamete ratios were not explored during artificial fertilizations in the present study. However, using common species to investigate correlation estimates between fertilization capacity and motility parameters could produce useful information regarding the fertilization potential of a particular batch of frozen sperm. This first requires knowledge of the optimal sperm to ovum ratio for maximum fertilization and is a common method employed in the aquaculture industry where obtaining eggs can be problematic (Moccia and Munkittrick, 1987).

Artificial fertilization using frozen-thawed sperm from a number of anuran species has resulted in fertilization and hatching, however the completion of metamorphosis has not been reported (Table 5). Artificial fertilization trials in the present study produced low fertilizations rates overall, with only 32.3 ± 2.9% cleavage in fresh sperm controls, and may have been a result of poor egg quality. However, despite the low fertilization rates, the proportion of cleaved embryos derived from fresh sperm did not differ significantly between cleaved embryos from frozen-thawed A. b. boreas sperm (17.6 ± 5.7%). Furthermore, there was no significant difference in the developmental rates or the proportion of tadpoles that completed metamorphosis between fresh or
frozen-thawed sperm. Although, the fertility of an F1 generation must be established before the entire artificial fertilization procedure can be deemed successful, these are encouraging findings for the generation of a functional amphibian GRB.

**Cryopreservation protocol optimization**

Sperm motility (and fertilization capacity in *A. b. boreas*) was recovered from all three species, however further exploration of protocol modifications to optimize and refine the freezing technique described herein, is warranted. In consideration of the wider parameters of cryopreservation methodology, the following modifications for future research should include: 1) standardized sperm concentration artificial fertilization trials to determine the influence of sperm density on freezing capacity. In the present study, sperm concentration was not adjusted to an established value prior to freezing. However, a number of reports in fish indicate that sperm density is an extremely important factor in sample preparation for cryopreservation and can directly influence post-thaw values (Dong *et al*., 2007). Furthermore, there is evidence to suggest that sperm density, and not dilution factor, determines motility in some fish species (Jing *et al*., 2009); 2) the incorporation of an extended range of CPA incubation temperatures (i.e. 0° C and 10° C) to regulate sperm cell permeation rates, and gauge the effect on post-thaw recovery; 3) the addition of chemical controls in artificial fertilizations to ascertain CPA toxicity effects on fertilization rates and embryonic development of fresh sperm compared to frozen-thawed sperm; 5) the investigation of an optimum sperm: egg fertilization ratio to better estimate fertilization rates of frozen-thawed sperm samples using motility assays alone; and, 6) determine the potential for premature sperm acrosome activation, either through exposure to a cryoprotectant solution or the freezing/thawing process.
Conclusions

The relatively novel technique of spermic urine cryopreservation is immensely valuable to the conservation of small and endangered amphibian populations, and the realization of a genome resource bank (GRB). The results of the present study demonstrate that an effective common spermic urine cryopreservation methodology can be developed in a model Bufonid (*Anaxyrus fowleri*) with successful application to species of conservation concern from different genera within the same family. We report the recovery of moderate post-thaw sperm motility parameters from the critically endangered Puerto Rican crested toad (*Peltophryne lemur*) and the threatened Southern Rocky Mountain boreal toad (*Anaxyrus b. boreas*). In addition, artificial fertilization using frozen-thawed *A. b. boreas* sperm and comparative analysis of embryonic development verified that post-thaw fertilization capacity was retained and developmental outcomes did not appear compromised by the freezing process.

This methodology demonstrates an encouraging inter-species transfer of technology through an effective cryopreservation technique that requires non-specialized and inexpensive equipment. Specifically, a slow-rate freezing method using either 5% or 10% DMFA or DMSO in a trehalose-based cryoprotectant solution, with a pre-freeze equilibration time of 10 min at 4° C, a cooling rate of 20-25° C min⁻¹ with a 40° C thaw for 5 seconds. The freezing method would be highly suited for use in zoos and research institutions operating under a limited budget, and could easily be applied in the field for the collection and cryopreservation of spermic urine samples from wild anuran populations.
To the best of the author’s knowledge, the present study represents the first application of cryopreservation techniques to anuran species of conservation concern, resulting in the successful production of offspring from the threatened boreal toad and the successful recovery of motility parameters from the critically endangered Puerto Rican crested toad; both target species of active captive-breeding and release programs. These results provide a platform for further refinement of spermic urine cryopreservation technology and the transfer of methodologies to other threatened anuran species.

**Acknowledgements**

This study was supported an Institute of Museum and Library Services National Leadership Grant (LG-25-09-0064-09) and a Memphis Zoo Conservation Action Network Award. The authors would like to thank the Mississippi Agricultural and Forestry Extension Service (MAFES), Omaha’s Henry Doorly Zoo, and the Colorado Division of Wildlife, in particular Harry Crocket, Ted Smith and Tom Mix and all the staff at the Native Aquatic Species Restoration Facility for accommodating our research. We are also grateful to Michael Robinson, Lindsay Bullock, Amanda Mullen and Andrew House for their help with the husbandry and care of the MSU boreal and Fowler’s toad colony.
References


212


CHAPTER VI
SPERMIATION INDUCTION, CRYOPRESERVATION AND POST-THAW FERTILITY IN THE CRITICALLY ENDANGERED MISSISSIPPI GOPHER FROG (*Lithobates sevosa*)

**Introduction**

The current amphibian conservation crisis requires urgent measures to counter the threat of extinction for a growing number of vulnerable species in the wild. Without immediate conservation measures, many species will be lost forever. Ecosystem-based *in-situ* conservation measures, including habitat preservation and restoration, whilst high priority strategies, are not always a viable option due to ongoing threats, political obstacles or total habitat loss (Hambler and Canney, 2004; Watson and Holt, 2001). Therefore, *ex-situ* conservation methods may offer the only hope of survival for many species on the brink of extinction (Ryder, 2000). Removal of threatened species from their native habitat provides insurance against loss in the wild and a means to facilitate population augmentation through captive breeding programs (CBPs) for eventual wild reintroductions (Hambler and Canney, 2004).

The critically endangered Mississippi (MS) gopher frog (*Lithobates sevosa*) is a distinct population segment of the dusky gopher frog (*Lithobates capito*) and is listed among the world’s 100 most endangered species by the International Union for the Conservation of Nature (IUCN, 2015). Over the past 50 years, *L. sevosa* has been
extirpated from much of its historical range within the longleaf pine ecosystem along the lower coast of Louisiana and Alabama, USA (Rorabaugh, 2005). Current population estimates are around 125 breeding adults located in one pond in Harrison County, MS, USA [MS Gopher Frog Recovery Team Meeting, 2012]. In addition to the destruction of the long-leaf pine habitat, recent urbanization of the surrounding area and irregularity in periodic prescribed burns have altered the hydrological period of the ephemeral ponds on which the MS gopher frog relies for explosive breeding events, induced by heavy rainfall (Thurgate, 2007). When the hydroperiod is shortened, water levels are insufficient and ponds are liable to desiccate before tadpoles can complete metamorphosis.

Environmental variability, coupled with genetic isolation leaves the Mississippi gopher frog population highly vulnerable to extinction through an extreme event, such as a disease epidemic, or habitat contamination. Under such circumstances, captive breeding programs become vital to secure the extant genetic diversity of these small populations for the successful recovery of the species. In response to the precarious situation of the last remaining wild *L. sevosa* population, between 2007 and 2010, a number of wild-caught adult gopher frogs were brought into captivity and distributed between four North American zoos to establish CBPs. However, the Mississippi gopher frog fail to exhibit natural mating behavior in captivity [pers. observ.] where the reproductive cues that stimulate breeding behavior in the wild are absent. Humidity and rainfall are likely the proximate causes of reproduction in *L. sevosa* and are associated with the activation of migration to breeding sites for the initiation of courtship and mating (Salthe and Mechan, 1974). Such environmental conditions are hard to emulate in captivity and as such, assisted reproductive technologies, such as exogenous hormone administration, and
artificial fertilization (AF), are essential for circumventing environmental stimuli to induce gamete release in captive gopher frogs.

ARTs have the potential to support and enhance breeding programs and boost the reintroduction efforts of captive species (Kouba and Vance, 2009). Founding *L. sevosa* adults are bred in captivity each year using hormone stimulation and AF, and a number of offspring per breeding pair are retained for future breeding [MS Gopher Frog Recovery Team Meeting, 2013]. The use of ARTs has been enormously beneficial for the recovery of *L. sevosa*, increasing the captive population to over 600 individuals housed at 14 zoos nationwide [MS Gopher Frog Recovery Team Meeting, 2013]. Not only does this provide an essential breeding population for future reintroduction programs but also makes available surplus individuals as research animals for the development of additional ARTs, such as gamete cryopreservation methods.

The successful development of a cryopreservation protocol would allow the establishment of a frozen repository of *L. sevosa* gametes which would be of enormous value to the genetic management of the captive breeding program. In addition to safeguarding valuable genetics, the effective captive population size could be increased through the exchange of gametes between breeding facilities, without compromising animal welfare or biosecurity, thereby providing a means to diversify small population genetics without housing additional animals (Frankham *et al.*, 2010; Kouba and Vance, 2009; Kouba *et al.*, 2013; Wildt, 2000). A GRB would also provide easy access to frozen sperm samples to coordinate artificial fertilizations synchronization. A major challenge in captive breeding is asynchronous gamete release, and the immediate availability of frozen sperm would be immensely helpful for artificial fertilization (Roth and Obringer, 2003).
In addition frozen sperm samples from different males would allow multiple paternity fertilizations of one egg mass: a highly useful technique for captive population genetic management.

Over the last two decades sperm cryopreservation protocols have successfully been developed in a number of common anuran species (Buchholz et al., 2004; Beesley et al., 1998; Browne et al., 1998; Browne et al., 2002; Mungano et al., 1998; Kaurova et al., 2006; Mansour et al., 2009ab; Mansour et al., 2010; Michael and Johnson, 2004; Sargent and Mohun, 2005) but it was only recently that a gamete repository for endangered anuran species became feasible. Historically, anuran sperm cryopreservation involved harvesting and macerating the testis: a technique not appropriate for endangered species. In recent years the successful cryopreservation of spermic urine collected non-invasively from live-animals has been reported in three different anuran species: *Rana pipiens* (Hopkins and Herr, 2007), *Rana temporaria* (Shishova et al., 2011), and *Pelophylax lessonae* (Uteshev et al., 2013). However, there are no reports in the literature of the successful application of spermic urine cryopreservation techniques to any endangered species. The recent development of a cryopreservation protocol with successful application across three Bufonid species (*Anaxyrus fowleri*, *A. b. boreas* and *Peltophryne lemur*) is a promising step towards an amphibian biobank and the potential transfer of a cryopreservation technique to different species (Langhorne et al., unpublished). In the face of ongoing rapid global population declines, efforts must be stepped up to establish effective cryopreservation techniques to safeguard the genetics of vulnerable anuran populations.
The key for cryopreservation technology to be a useful technology is first being able to characterize the spermiation response for the collection of gametes (Kouba et al., 2003). A number of protocols have been developed for the minimally-invasive collection of gametes from male anurans, through exogenous hormone administration and release of gametes in urine (spermic urine). Two routinely used exogenous hormones for the induction of spermiation are human chorionic gonadotropin (hCG) and gonadotropin releasing-hormone agonist (GnRHa) (Kouba et al., 2012). A combined hormone dose of hCG and GnRHa has been shown to be successful for the stimulation of sperm release in the Mississippi gopher frog (Kouba et al., 2011).

The transfer of a cryopreservation method effective in three Bufonid species, to a critically-endangered Ranid species was the focus of this study. The overall aim was to induce spermic urine production using an established hormone regimen for the collection of gametes from L. sevosa to verify a successful sperm cryopreservation method for the critically endangered Mississippi gopher frog. Specific objectives were to: 1) characterize spermiation response over time to exogenous hormone administration; 2) compare the quality and quantity of fresh and frozen-thawed spermatozoa; 3) evaluate the fertilization capacity of frozen-thawed spermatozoa; 4) compare embryonic development and timing to metamorphosis of embryos derived from fresh and frozen-thawed spermatozoa artificial fertilization.

**Materials and Methods**

All animal procedures were conducted following review and approval by the Mississippi State University Institutional Animal Care and Use Committee.
Animals

The captive colony of adult Mississippi gopher frogs were housed in ventilated polycarbonate containers (30 cm L x 46 cm W x 66 cm H) with access to water and shelter. Frogs were maintained at 20–23°C and provided with standard fluorescent lights on a natural day/night cycle. Mixed-sex groups of three or four individuals were housed per container and offered a variety of food items including crickets, wax worms, and mealworms, three times per week. Crickets were gut-loaded with Repashy SuperLoad© (Repashy Ventures Inc., CA, USA) supplement and calcium dusted prior to being fed out.

Study 1: Gamete induction, collection and quantification following exogenous hormone treatment in male L. sevosa

Spermic urine induction, collection and analysis

For the duration of the hormone trial, sexually mature male Mississippi gopher frogs (5-6 years old; n=17) were maintained in plastic containers (35 cm L x 20 cm W x 13 cm H) holding aged tap water (2 cm depth) to ensure continuous urine production. Prior to hormone administration a urine sample (T₀) was obtained from each frog to determine the presence or absence of sperm. To obtain urine, males were gently removed from their holding containers and held around the waist with their hind limbs spread apart by the thumb and index finger to enable access to the cloacal opening. A fire-polished catheter was inserted 1 cm into the cloaca to stimulate urination. Catheter contents were expelled into a 150 mm petri dish and collected into a sterile 1.5 mL Eppendorf tube.

Spermiation was induced by intraperitoneal (IP) injection of saline containing a combination of 500 IU human Chorionic Gonadotropin (hCG; cat# C1063; Sigma-Aldrich, St. Louis, Missouri, USA) and 15 μg LHRH analog ([des-Gly₁₀, D-Ala⁶]-LH-
RH ethylamide acetate [cat# L4513; Sigma-Aldrich) in a total volume of 215 µl. Spermic urine was collected, as described above, every 30 minutes between 0.5 and 2.5 h post-hormone administration (PA). A control group of males (n=10) was administered a 200µl IP injection of sterile saline. Samples were analyzed by placing a 10 µl aliquot of spermic urine onto a glass slide under a 400x objective on a phase-contrast microscope (Olympus CX41) and counting the first 100 sperm. Spermic urine variables evaluated included: Total Motility ([TM]; = flagellum movement) and Forward Progressive Motility ([FPM] = forward progressive movement as a proportion of total count). Sperm concentration was measured by inactivating motility in a 1:10 dilution of PBS and counting using a Neubaeur haemocytometer to obtain an average sperm concentration mL⁻¹. Spermic urine samples were held either in a refrigerator at 4°C or cryopreserved in liquid nitrogen, prior to artificial fertilization trials.
Ovulation and egg deposition

Table 7  Hormone treatments for induction of ovulation and egg deposition in *L. sevosa* females treated with one of four exogenous hormone regimens

<table>
<thead>
<tr>
<th>Female ID</th>
<th>LHRHa (µg/g)</th>
<th>MET (µg/g)</th>
<th>hCG (IU/g)</th>
<th>hCG Prime (IU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2, 3</td>
<td>0.4</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.4</td>
<td>-</td>
<td>13.5</td>
<td>-</td>
</tr>
<tr>
<td>5, 6</td>
<td>0.4</td>
<td>-</td>
<td>13.5</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Each numbers (1-6) represents one female

Females from a separate study were utilized for the purposes of artificial fertilization in this experiment. Four different combinations of exogenous hormones were trialed to initiate ovulation in *L. sevosa*. Females 1, 2 and 3 were administered an ovulatory dose of GnRHa and a dopamine antagonist (MET=Metaclopramide) combined. Females 4, 5 and 6 were also administered a combined ovulatory dose of GnRHa with hCG. In addition to the GnRHa/hCG ovulatory dose females 5 and 6 received a priming dose of hCG 96 h and 24 h in advance of the ovulatory dose.

Hormone treatments were calculated by per gram body weight (BW) and included: 1) a combined ovulatory dose of GnRH (0.4 µg g⁻¹) plus hCG (13.5 IU g⁻¹); 2) the same treatment as number 1, except 96 h and 24 h in advance of the combined ovulatory dose the females were primed with 3.7 IU g⁻¹ hCG; and 3) a combined ovulatory dose of GnRH (0.4 µg g⁻¹) plus 10 µg g⁻¹ of the dopamine antagonist metoclopramide (cat# M0763; Sigma-Aldrich). Eggs were expressed by holding the female anterior to the rear legs using the thumb and forefinger above a 150 mm petri dish. The cloaca was opened up by gently inserting a rounded plastic probe (Coplan © Diagnostics; Cat# COP-S1) and lifting slightly to the side while simultaneously
squeezing lightly downwards from the forearms to the rear legs to facilitate egg passage towards the cloaca. Once eggs were visible through the cloacal opening, the probe was slowly removed and eggs (32.0 ± 1.0 per dish) were deposited into 150 mm diameter petri dishes.

**Study 2: Spermic Urine Cryopreservation**

Preliminary sperm freezing trials in *L. sevosa* males were based on methodologies that proved successful in three Bufonid species. Males (*n*=6) were induced to spermiate using the hCG protocol previously outlined (Methods: 6.6.2). Spermic urine samples collected over the 2.5 h period following hormone administration were pooled for each male. Sperm motility parameters were analyzed immediately prior to cryopreservation, so as to provide standardized spermic urine quality parameters for accurate evaluation of post-thaw motility recovery.

Spermic urine samples selected for cryopreservation met the following quality standards: ≥80% total motility; ≥ 10% forward progressive motility; motility quality [FPM-Q] ≥ 2 ([FPM-Q] = a subjective scale from 0 (no movement) to 4 (very rapid forward movement)); and, sperm concentrations ≥ 0.8 x 10^6 per ml. Spermic urine aliquots (100 µl) were diluted 1:1 in chilled cryoprotectant solution containing 0.5 M trehalose (cat# T5251; Sigma-Aldrich) diluent with the addition of 10% [(v/v) final concentration] dimethyl formamide ([DMFA] cat# D4551; Sigma-Aldrich). The cryoprotectant solution were prepared at double strength of final concentrations, prior to mixing with spermic urine aliquots (100 µl) at a ratio of 1:1. The resulting cryosuspensions (200µL) were equilibrated at 4 °C for 10 min before loading into 0.25 mL plastic freezing straws [Minitube International®, Germany] (x 8 straws/CPA) and plugging with Critoseal®.
Straws were placed in a Styrofoam freezing box on a rack 10 cm above the level of liquid nitrogen and held in vapor for 10 min before plunging into liquid nitrogen.

**Study 3: Artificial fertilization using fresh and frozen-thawed sperm**

Frozen sperm samples for artificial fertilization trials were collected from a subset of males ($n=9$) of known high quality pre-freeze spermic urine samples. Upon egg deposition, frozen sperm samples were removed from liquid nitrogen storage and rapidly thawed by immersion in a 40 °C water until all visible ice crystals had melted (~ 5 s). Straw contents (200 µl) were expelled into a small plastic dish and a 10 µl aliquot was activated by a 1:10 dilution with aged tap water for post-thaw analysis of sperm motility parameters, concentration and osmolality (mOsm kg$^{-1}$). The remaining 190 µl frozen-thawed sperm was used to fertilize two separate egg dishes by pipetting half the sperm (95 µl) evenly over each egg mass followed by 900 µl aged tap water to induce sperm activation. Control dishes were fertilized with 100 µl fresh sperm from the corresponding male, such that each male served as its own fresh control. Fresh sperm was maintained at 4°C and extended in non-spermic T₀ urine, collected from each male prior to hormone administration, to a standard concentration (965 ± 97 sperm/egg). Parthenogenic control dishes ($n=3$) were mixed with 100 µl non-spermic urine to control for cleavage activated by means other than spermatozoa. After 5 min the dishes were flooded with aged tap water until the egg mass was fully covered. Artificial fertilization was performed within 3 min of oocyte recovery for both fresh sperm controls (x 3 fertilization dishes/female) and frozen-thawed sperm (x 4 fertilization dishes/female).
Study 4: Embryonic development and metamorphosis

Cleavage rates were assessed by counting the number of 2-4 cell embryos under a 10x objective on a dual-power stereo microscope (Omano, OM4713), 4–6 h post-fertilization. Developmental stages were monitored daily and any non-fertilized or dead embryos were carefully removed from each fertilization dish. On hatching (Gosner stage 20), all tadpoles were transferred into larger plastic tubs (28 cm L x 20 cm W x 28 cm H) and fed ground tropical fish flakes (Tetra®, United Pet Group, Blacksburg, VA) once per day and algae pellets (Tetra®) ad libitum. Partial water changes (30-50%) were performed every 48 h and water temperature was recorded daily using digital thermometers (Coralife Products, Franklin, WI, USA). At Day 60 post-fertilization, tadpoles produced from both frozen-thawed and fresh sperm were separately pooled and a sub-set (n=60) of tadpoles from each group (fresh or frozen) were randomly allocated to 3 plastic tubs (45 cm L x 29 cm W x 30 cm H) each with a density of 20 tadpoles per tub to continue development. Upon emergence of front limbs (Gosner stage 42), tadpoles were removed into smaller tubs (50 cm L x 25 cm W x 35 cm H) containing 1 L aged tap water. Each tub was placed at a 20° degree angle such that the water pooled only in one half of the tank to facilitate exit from the water on full limb emergence. On completion of metamorphosis (characterized by complete tail regression; Gosner stage 46), the date, weight (g) and SVL (mm) of each individual was recorded.

Statistical analysis

Assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk and Levene’s tests, respectively. Percentage data were arcsine transformed using the transformation $\sin^{-1}(\sqrt{x})$ before further analysis. Differences between individual
means were analyzed using independent or paired samples t-tests. To determine the
effects of cryopreservation on sperm motility parameters (TM and FPM) paired samples
tests were used to compare the motility parameters of fresh samples to the motility
recovered from frozen-thawed samples for each male. All developmental stage (cleavage,
tadpole and metamorph) and metamorph body characteristics (weight and SVL)
comparisons between fresh and frozen-thawed artificial fertilization trials were analyzed
using independent samples t-tests. Significance was established at $P \leq 0.05$. All statistical
analysis was performed in SAS Version 9.4 (Cary, North Carolina, USA). All data are
expressed as mean ± SEM.

Results

Study 1: Gamete induction, collection and quantification following exogenous
hormone treatment

Spermiation response

Urine was obtained from male gopher frogs on at least 96% of collection
attempts. Mean osmolality of urine was 45.3 ± 4.8 mOsm kg$^{-1}$ and urine sample volumes
ranged from 30 µl to 1000 µl. Sperm was absent from all T$_0$ urine samples collected from
each male prior to the administration of exogenous hormones or sterile saline. Control
animals injected with sterile saline did not produce sperm ($n=0/10$) at any of the
collection time-points (0.5, 1.0, 1.5, 2.0 and 2.5 h PA).

Exogenous hormone treatment with a combination of hCG and GnRHa induced a
spermiation response in 100% ($n=17$) of injected $L. sevosa$ males (Figure 23). However,
response varied across collection time-points, meaning that some males did not release
sperm at certain time-points. Overall however, the response to hormone treatment was
high, between 70% - 90% across the 2.5 h collection period (Figure 23). Sperm production was initiated in 82% (n=14/17) of male frogs within 30 min PA, when peak sperm production was $3.4 \pm 1.1 \times 10^6$ mL$^{-1}$ (Figure 24).

Sperm was released by 94% - 100% of frogs between 1 – 2 h PA but had decreased to 71% by the final collection time-point (Figure 23). Sperm concentration decreased precipitously across the subsequent time-points and by 2.5 h PA had reduced to $0.4 \pm 0.6 \times 10^6$ mL$^{-1}$ (Figure 24). Sperm production between individual males was highly variable across all time-points, ranging from $0.01 \times 10^6$ mL$^{-1}$ to $17 \times 10^6$ mL$^{-1}$ at 0.5 h PA and from $0.08 \times 10^6$ mL$^{-1}$ to $2.0 \times 10^6$ mL$^{-1}$ at 2.5 h PA (Figure 24).

Sperm motility parameters [TM, FPM] of hormone-treated L. sevosa were much less variable between individual males at each time-point. Total motility ranged from $84.0 \pm 2.4\%$ to $87.0 \pm 4.7\%$ with mean forward progressive motility (FPM) of $25.0 \pm 6.2\% - 30.0 \pm 5.1\%$ across the 2.5 h collection period (Figure 25).
The number of male MS gopher frogs \((n=17)\) exhibiting spermatozoa in urine samples collected at 5 time-points (0.5, 1, 1.5, 2 and 2.5 h) post-hormone administration (PA) of a combination hormone dose comprising 500 IU hCG and 15 µg GnRHa. Responders were classified as individuals producing spermic urine at each time-point PA. Response to hormone treatment was 70-100% across all time-points. Values are expressed as percentages of the total number of males producing spermic urine at each collection time-point.
Percentage of spermatozoa exhibiting Total Motility (TM) and Forward Progressive Motility (FPM) from urine samples collected from male *L. sevosa* (*n*=17) at 5 time-points (0.5, 1, 1.5, 2 and 2.5 h) post-hormone administration (PA) of 500 IU hCG + 15µg GnRHa. Spermatozoa TM and FPM ranged from 84 ± 2.4% - 87 ± 4.7% and 25 ± 6.2% - 30 ± 5.1% respectively, across the collection period. Values are expressed as untransformed means of the total number of male responders exhibiting motile sperm at each collection time-point ± SEM.

**Study 2: Sperm motility recovery from cryopreservation**

The following data refers to frozen-thawed sperm samples from a preliminary freezing trial of *L. sevosa* (*n*=6) spermatozoa to ascertain the feasibility of transferring a cryopreservation technique developed in the Bufonidae, to a critically-endangered Ranid species.

Figure 25 shows post-thaw motility and forward progressive motility of frozen-thawed *L. sevosa* sperm. Overall, frozen-thawed sperm motility was significantly reduced from 90.0 ± 3.6% in fresh sperm samples to 72.5 ± 2.5% in frozen-
thawed sperm samples (Figure 26). However, the cryopreservation process had no significant effect on the proportion or quality of forward motile *L. sevosa* spermatozoa, with 32.1 ± 7.2% FPM at a quality ranking of 2.3 ± 0.4, compared to 28.5 ± 8.7% FPM at a quality ranking of 2.5 ± 0.6 of frozen-thawed and fresh sperm respectively (Figure 26).

Figure 26  Effect of freeze-thawing on the motility parameters of spermatozoa from *L. sevosa*

Spermatozoa motility parameters [TM and FPM] of fresh, and frozen-thawed spermatozoa from *L. sevosa* following cryopreservation in a 0.5 M trehalose-based cryoprotectant solution comprising a final concentration of 10% DMFA. The total sperm motility was significantly reduced following cryopreservation, in relation to fresh sperm (*P*<0.05). However, there was no significant difference (*P*>0.05) in the proportion of fresh and frozen-thawed sperm exhibiting forward motility. abValues superscripted with the same letter do not differ significantly (*P*>0.05) and are expressed as untransformed means ±SEM.

**Study 3: Artificial fertilization using fresh and frozen-thawed sperm**

A sub-set of male gopher frogs (*n*=9) was selected for artificial fertilization (AF) trials based on their history of consistently high quality sperm production according to
the sperm quality criteria described in the methods above. Sperm samples from the subset of males were cryopreserved opportunistically and as such, had been held in liquid nitrogen for varying lengths of time, ranging between 48 h - 13 months prior to thawing. Figure 27 shows the mean sperm motility and proportion of forward motile spermatozoa recovered from frozen-thawed *L. sevosa* sperm samples of males (*n*=9) used in AF trials.

Paired samples *t*-tests indicated an overall significant difference in post-thaw sperm motility (*t*=4.77; *P*<0.001), FPM (*t*=4.54; *P*<0.001) and FPM-Q (*t*=4.26; *P*<0.001) in relation to the sperm motility parameters of the corresponding fresh sample (Figure 27 and Figure 28). The percentage of post-thaw sperm motility was variable, ranging between 50% - 85% across the 9 male frogs (Figure 27). This equates to a motility recovery rate (frozen-thawed sperm motility/fresh sperm motility x 100) of between 51%- 92%, relative to pre-freeze motility parameters (Figure 27). Post-thaw sperm FPM ranged between 7%- 20% (18%- 100% recovery relative to corresponding fresh sperm FPM; Figure 27) and frozen-thawed sperm FPM-Q was between 1.4 ± 1.6– 2.2 ± 0.8 (Figure 28).

Notably, frozen-thawed sperm recovered from Male E, which had been maintained in cryostorage for the longest period (> 12 months), exhibited among the highest recovered sperm motility parameters with post-thaw motility parameters of 91% total motility and 100% FPM recovery, relative to the fresh sperm sample prior to freezing. Sperm concentrations of frozen-thawed samples ranged between 0.05 - 0.25 x 10⁶ mL⁻¹ and osmolality of 58.4 ± 4.2 mOsm kg⁻¹ across the nine male *L. sevosa*.

Eleven breeding pairs were utilized for the fertilization trial (*n*=9 males; *n*=6 females). No cleavage was observed in the parthenogenic control dishes. Table 8 shows
the total number of eggs per breeding pair and the cleavage rates of embryos fertilized using fresh (control) and frozen-thawed sperm samples. There was no significant difference in the cleavage rates of embryos derived from fresh and frozen-thawed spermatozoa with $72 \pm 6\%$ and $55 \pm 8\%$, of cleavage in fresh and frozen-thawed artificial fertilizations, respectively ($t_{20}=1.47; P=0.15$; Table 8).

![Graph showing percentage motility of spermatozoa from fresh spermic urine samples (white bar), and percent TM (striped bar) and FPM (grey bar) recovered from frozen-thawed male L. sevosa spermatozoa.](image)

Figure 27  Percentage motility of spermatozoa from fresh spermic urine samples (white bar), and percent TM (striped bar) and FPM (grey bar) recovered from frozen-thawed male L. sevosa spermatozoa

Percentage fresh spermatozoa Total Motility (TM) (white bar) from spermic urine samples of *L. sevosa* (*n*=9) prior to freezing in a CPA comprising 10% (v/v) DMFA in a 0.5 M (w/v) trehalose cryoprotectant. Frozen-thawed spermatozoa TM (striped bar) and Forward Progressive Motility (FPM; grey bar) were evaluated on thawing. Post-thaw sperm motility ranged between 50% - 85% with the proportion of forward motile sperm between 7% - 20% across the nine male toads. Each letter (A - I) represents one male. Fresh TM values are expressed as percentages and frozen-thawed TM and FPM values are expressed as means of the total number of sperm straws thawed for each male (*n*= 5 - 10 straws/male).
Figure 28 The quality of forward motile *L. sevosa* sperm before (white bar) and after (grey bar) cryopreservation in a DMFA-based cryoprotectant

The quality of forward progressive motility [FPM-Q] of fresh (white bar) and frozen-thawed (grey bar) *L. sevosa* (*n*=9) sperm samples cryopreserved in a CPA comprising 10% (v/v) DMFA in a 0.5 M (w/v) trehalose cryoprotectant. Post-thaw FPM-Q ranged between 1.4 ± 1.6– 2.2 ± 0.8 across the nine male frogs. Each letter (A - I) represents one male. Fresh FPM-Q values are expressed as the quality ranking for the fresh sperm sample and frozen-thawed FPM-Q values are means of the total number of sperm straws thawed for each male (*n* = 5 -10 straws/male).
Table 8  Total number of *L. sevosa* eggs from each breeding pair and the percentage cleavage rate after artificial fertilization with either fresh or frozen-thawed spermatozoa

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
<th>Control</th>
<th>Frozen</th>
<th>Control</th>
<th>Frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>67</td>
<td>73</td>
<td>75</td>
<td>58</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>102</td>
<td>124</td>
<td>75</td>
<td>62</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>107</td>
<td>118</td>
<td>63</td>
<td>53</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>87</td>
<td>123</td>
<td>94</td>
<td>80</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>56</td>
<td>49</td>
<td>89</td>
<td>37</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>46</td>
<td>68</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>108</td>
<td>169</td>
<td>81</td>
<td>5</td>
</tr>
<tr>
<td>G</td>
<td>3</td>
<td>101</td>
<td>154</td>
<td>83</td>
<td>45</td>
</tr>
<tr>
<td>G</td>
<td>4</td>
<td>125</td>
<td>139</td>
<td>46</td>
<td>37</td>
</tr>
<tr>
<td>H</td>
<td>6</td>
<td>108</td>
<td>195</td>
<td>73</td>
<td>98</td>
</tr>
<tr>
<td>I</td>
<td>6</td>
<td>81</td>
<td>129</td>
<td>93</td>
<td>90</td>
</tr>
</tbody>
</table>

Mean ± SEM  

|        | 90 ± 7 | 122 ± 13 | 72 ± 7 | 55 ± 8 |

The total number of eggs and cleavage rate of all male/female *L. sevosa* breeding combinations for artificial fertilization using fresh and frozen derived spermic urine. There was no significant difference (*P* > 0.05) in the cleavage rate of embryos derived from frozen-thawed sperm, relative to fresh sperm artificial fertilization.

**Study 4: Embryonic development and metamorphosis**

Water temperature was recorded daily (temperature range: 17.7 ± 0.6°C – 20.0 ± 0.6°C) and there was no significant difference in temperature between tadpole tanks (*P* > 0.05). Embryonic development was monitored through metamorphosis, and on full tail regression, weight and SVL was recorded. The average weight of metamorphs
(tadpoles completing full tail regression) from fresh sperm AF trials was significantly lower \((t_{58} =-1.89; P=0.03)\) than the weight of metamorphs from frozen-thawed sperm AF trials \((4.7 \pm 0.2 \text{ g and } 5.2 \text{ and } 0.2 \text{ g, respectively})\). There was no significant difference \((t_{50}=0.1; P=0.92)\) between the SVL of metamorphs from both fresh and frozen-thawed AF trials \((33.1 \pm 0.4 \text{ mm, for both frozen-thawed and fresh sperm derived metamorphs})\).

Although there was no significant difference in the cleavage rate, significantly more embryos reached tadpole stage from fresh in relation to frozen-thawed sperm AF trials \((t_{20}=2.50; P=0.02; \text{ Figure 29})\). Tadpoles from frozen-thawed sperm AF trials initiated and completed metamorphosis between 6 – 12 months PF, and 5- 9 months post-fertilization, for fresh and frozen-thawed artificial fertilization trials, respectively. The overall percentage of tadpoles completing metamorphosis was 55% from fresh sperm AF trials and 70% from frozen-thawed sperm AF trials (Figure 29). Only one tadpole from fresh sperm AF trials did not metamorphose. Although only select developmental stages were recorded, similar rates of development were observed between both the fresh and frozen-thawed spermatozoa artificial fertilizations (Figure 30).
Figure 29  Percentage of fertilized embryos to reach cleavage, tadpole and metamorph stage following artificial fertilization with fresh and frozen-thawed *L. sevosa* sperm

The proportion of fertilized embryos to reach cleavage, tadpole and metamorph stage following cryopreservation in a 10% (v/v) DMFA and 0.5 M (w/v) trehalose cryoprotectant, compared to fresh sperm controls. There was no significant difference (\(P>0.05\)) in the cleavage rate of embryos derived from fresh sperm and frozen-thawed sperm artificial fertilizations. However, significantly more embryos fertilized with fresh sperm progressed to tadpole stage (\(P<0.05\)). Cleavage and tadpole data are mean values of fertilization dish replicates per breeding pair ± SEM. Metamorph data represents the percentage of tadpoles that metamorphosed from the pooled sub-set of tadpoles (\(n=60\)) and is therefore represented as a single value per treatment [fresh/frozen-thawed].

\(\text{ab}\) Values superscripted with the same letter within a developmental stage are not significantly different (\(P>0.05\)).
Figure 30  Embryonic development of eggs fertilized using frozen-thawed *L. sevosa* sperm

Developmental stages of *L. sevosa* embryos fertilized using frozen-thawed sperm. From top left: (A) Cleavage: 4-5 h post-fertilization [PF] (Gosner Stage 2) (B) 16-cell: 7-8 h PF (Gosner Stage 6) (C) Tail-Bud: 96 h PF (Gosner Stage 17) (D) Muscular Response: 5 d PF (Gosner Stage 18) (E) Heartbeat/Hatching: 8 d PF (Gosner Stage 20) (F) Tail Fin Circulation: 10 d PF (Gosner Stage 22) (G) Tadpole: 12 d – 87 d PF (Gosner Stage 25) (H) Juvenile: 92 d PF. Similar developmental rates were recorded in fresh and frozen-thawed sperm artificial fertilizations.

**Discussion**

The present study describes the preliminary stages in the development of assisted reproductive technologies (ARTs) for a critically endangered anuran species. The current surplus captive population of the Mississippi gopher frog, *Lithobates sevosa*, provides a valuable resource for developing and refining ARTs to enhance reproductive output and facilitate the genetic management of the captive *L. sevosa* breeding program. Ultimately, an augmented population and maintenance of genetic variability could improve the prospect of successful *L. sevosa* reintroductions to the wild. Furthermore, the
technologies developed herein may have potential applications to other threatened and endangered anuran species.

The *L. sevosa* breeding program has already greatly benefitted from the use of ARTs to substantially increase the captive population size through exogenous hormone therapy and artificial fertilization. The combined exogenous hormone treatment of hCG and GnRHa administered in the present study, originally developed by Kouba *et al.* (2011), induced spermiation in 100% of hormone stimulated male frogs. Sperm production was rapid and within 0.5 h post-hormone administration (PA), 82% of male gopher frogs reached peak sperm production with a concentration of $3.4 \times 10^6$ mL$^{-1}$. Over 80% of hormone-treated male gopher frogs continued to produce sperm up to 2.5 h PA, and sperm motility parameters remained consistent across the 2.5 h collection period. However, sperm concentrations dropped dramatically by 24 h PA to $0.4 \times 10^6$ mL$^{-1}$. Therefore, it would be recommended to collect spermic urine from the Mississippi gopher frog every 15 – 30 mins following hormone administration, in order to maximize the sample volume obtained from each male. The rapid onset of sperm release corresponds with the reproductive strategy of *L. sevosa*, which is characterized by explosive breeding and dictated by heavy seasonal rain events (Rorabaugh, 2005). This strategy is of particular use in artificial fertilization as *L. sevosa* can be injected with hormones *after* observing eggs in the cloaca of the female, ensuring synchronization of gamete deposition. This is also facilitated by the fact that female gopher frogs generally do not oviposit spontaneously, and require eggs to be manually squeezed from the cloaca [pers. observ.]. In contrast, the spermiation response of several Bufonid species, including *Anaxyrus baxteri, A. b. boreas* and *A. fowleri* (Browne *et al.*, 2006; Kouba *et al.*, 2011)
238

al., 2009; McDonough et al., 2015) is much slower, requiring toads to be injected with hormones several hours in advance of anticipated egg deposition, to coincide with peak sperm production. The species-specific discrepancy in spermiation profiles serve to highlight the importance of regular urine collections when developing hormone protocols in novel species, which should begin soon after hormone administration to ensure that peak sperm production is not overlooked.

In conjunction with exogenous hormone therapy and artificial fertilization, sperm cryopreservation is a valuable addition to the conservation tool box. The high rate of post-thaw motility recovery in preliminary freezing trials in the present study (73%) is highly encouraging and should provide momentum for the establishment of a frozen repository of L. sevosa gametes. Sperm cryopreservation, in addition to an active captive breeding program, would not only afford insurance against loss in the wild, but also insurance against the loss of important founding population genetics in captivity. One of the primary objectives of a successful CBP is ensuring genetic diversity is retained, which is most effectively achieved through maintenance of a large population (Frankham et al., 2010). However, limited space and resources restrict the population size that can realistically be accommodated in captivity (Kouba et al., 2013). The careful management and maintenance of genetic diversity is particularly important with regards to small captive populations, as a restricted gene flow can promote inbreeding depression and an accumulation of deleterious mutations, thus lowering survival rates (Boakes et al., 2007; Ficetola et al., 2011; Frankham et al., 2010). Furthermore, the longer a population remains in captivity, the more liable it becomes to genetic adaptation, which can occur in
several generations and compromise a species’ ability to persist in their natural environment on reintroduction (Ficetola et al., 2011; Frankham, et al., 2010).

Genetic adaptation to captivity occurs through both natural and artificial selection on the organism in the captive environment, and has been documented in fish, insects and amphibians (Frankham and Loebel, 1992; Heath et al, 2003; Kraaijeveld-Smit et al, 2006). For example, captive female Chinook salmon (Oncorhynchus tshawytsha) exhibited reduced reproductive success and laid smaller egg masses, relative to wild populations (Heath et al, 2003). In Drosophila melanogaster, a period of 12 months in captivity - the equivalent of eight generations - caused rapid genetic adaptation and a significant reduction in reproductive fitness, compared to their wild counterparts (Frankham and Loebel, 1992). It is unclear how long-term captive breeding affects fitness attributes in the amphibians (Kraaijeveld-Smit et al., 2006). A review of the literature returned a single report documenting a loss of fitness through genetic adaptation to captivity in the Mallorcan midwife toad (Alytes muletensis). A. muletensis tadpoles maintained in captivity for more than eight generations exhibited slower developmental rates and a loss of genetic variation, with an accompanying reduction in anti-predator defensive traits (Kraaijeveld-Smit et al., 2006). Further investigation into the possible effects of adaptation to captivity in the anurans is warranted, particularly in light of the potential implications for reintroduction strategies.

The best approach to minimizing genetic adaptation is thought to be a reduction in the number of generations that a species spends in captivity (Williams and Hoffman, 2009). However, with persistent global environmental threats, this option may not be viable for the majority of captive anuran species awaiting reintroduction. For species that
must remain in captivity, minimizing generations may take on two approaches – delayed reproduction of live animals and germplasm cryopreservation (Williams and Hoffman, 2009). Delayed reproduction is likely to be effective only in long-lived and highly fecund species and, therefore, has limited application across the anurans. Cryopreservation, however, provides a means of maintaining biodiversity and reducing adaptation to captivity through the introduction of frozen gametes, either from previous generations or potentially the immigration of gametes from wild-caught males into captivity through ex-situ sperm collection and cryopreservation.

A cryopreservation protocol is ultimately considered successful only if cryopreserved sperm fertilizes eggs and produces offspring. The present study demonstrated the development of an effective cryopreservation protocol for one of North America’s most endangered species. Artificial fertilization trials confirmed that fertilization capacity was retained in frozen-thawed *L. sevosa* sperm and the resulting embryonic development appeared unimpeded. Furthermore, the successful completion of metamorphosis by tadpoles derived from frozen-thawed sperm with equivalent or greater body parameter measurements [weight and SVL] as tadpoles derived from fresh sperm, indicates developmental competency and a typical growth pattern. However, as with any novel technology, caution should be exercised and further investigation of any long term reproductive or genetic effects in the F1 generation of offspring is warranted.

The recovery of sperm functional capacity from frozen-thawed samples and the production of offspring has enormous potential to enhance the genetic management of the *L. sevosa* CBP and secure extant genetic diversity. Moreover, the relatively basic and low cost freezing equipment would not only be a viable option for zoos and other institutions
operating within a tight budget, but could easily be transported to the field for spermic urine collections *in-situ*. This paper represents the first successful application of cryopreservation techniques to spermic urine collected non-invasively from a critically endangered amphibian, with the production of live young. These outcomes validate a freezing protocol previously established in three Bufonid species, suggesting the potential wider application of this methodology across the anura. The results presented in this paper provide the impetus for further advances in anuran sperm cryopreservation for the realization of a frozen gamete repository for endangered species.

**Acknowledgements**

This study was supported by funding from the Institute of Museum and Library Services, Grant # LG-25-09-0064-09. The authors are grateful to the Mississippi Forestry and extension Service. We also wish to thank the following student workers at Mississippi State University who assisted with the care and husbandry of the captive Mississippi gopher frog colony: Amanda Mullen, Andrew House, Mathew Christiansen, Michael Robinson and Christy Steadman. We are also grateful to staff at the Memphis Zoo and Racine Zoo for providing the animals used in this study.
References


MS gopher frog recovery team meeting. Jan. 9th 2011. Harrison County, MS, USA.


244


CHAPTER VII
LINKING IN SITU AND EX SITU POPULATIONS OF A DECLINING TOAD SPECIES (ANAXYRUS BOREAS BOREAS)

Introduction

Captive assurance colonies, are increasingly being recognized as necessary actions in the preservation efforts of species of conservation concern. Captive breeding typically involves the removal of a founder population from the wild for rearing and propagation, with the goal of reintroducing a sustainable population in the future (Hambler and Canney, 2004). Recent estimates suggest that roughly 950 amphibian species are currently in need of ex-situ conservation strategies (Bishop et al., 2012), with priority targets being small or declining populations that are likely more at risk of extinction (Frankham et al., 2010).

Previously widespread and locally common species such as the boreal toad (Anaxyrus boreas boreas) are declining in abundance and disappearing from substantial parts of their range in the North-western United States (Koch and Peterson, 2005). The boreal toad is typically found within federal and state protected areas of the Rocky Mountain National Park and habitat destruction along with the infectious chytridiomycosis disease, have been cited as the primary causes of boreal toad declines (Carey, 1993; Muths and Corn, 2000). Furthermore, fragmentation of native rangeland has resulted in a number of small populations, such as the geographically isolated
Southern Rocky Mountain boreal toad (SRMP). This population is thought to represent an independently evolving lineage (Goebel, 1996; Hammerson, 1999). However, genetic analysis has yet to confirm the SRM boreal toad as a distinct population segment and its current status is listed as ‘warranted but precluded’ from US federal listing, and near threatened by the International Union for the Conservation of Nature (IUCN) (Greenwald et al., 2011; IUCN, 2015).

Recent estimates suggest that around 100,000 breeding adults, comprising 25 distinct populations, remain at fifty breeding sites in just seven localities in the high alpine elevation zones (above 2000 m) of the Southern Rocky Mountains (Carey, et al., 2005; Loeffler et al., 2001). Historically, the proximity of numerous boreal toad breeding sites across the Southern Rocky Mountains would have facilitated genetic exchange and the formation of new populations (Loeffler et al., 2001). However, the disappearance of entire populations from previously occupied breeding sites currently limits dispersal ability, which in turn restricts gene flow between fragmented populations (Frankham et al., 2010; Loeffler et al., 2001). It is thought that only two or three of these populations reproduce successfully each breeding season and the remaining breeding populations are small (typically between one and five clutches laid per year) (Corn, et al., 1997; Loeffler et al., 2001).

Recovery efforts were initiated in 1995 to preserve existing boreal toad habitat, restore population sizes, and extend their range (Carey et al., 2005). The Colorado Parks and Wildlife’s Native Aquatic Species Restoration Facility (NASRF) has been actively involved in restoration efforts of the boreal toad at their captive breeding facility in Alamosa, CO, for over a decade [T. Smith, pers. comm.]. The NASRF captive breeding
population represents a priority group of Southern Rocky Mountain boreal toad populations that ensures maximal genetic diversity (Loeffler et al., 2001). Breeding populations at NASRF are housed in groups according to the region from which the original founding population was obtained. Therefore, strict management strategies are required to maintain the genetic diversity of each population segment. However, the reproductive output from each breeding population is low and can markedly vary each year, often resulting in complete breeding failure of individual populations over successive breeding seasons [NASRF staff, pers. comm.]. Inconsistent breeding coupled with an ageing population could compromise the boreal toad CBP through the loss of valuable founder individuals.

Fundamental to the overall success of a CBP is the careful management and maintenance of genetic diversity, which is particularly important with regards to small and declining populations (Frankham et al., 2010). As gene flow becomes restricted over time, reduced allelic diversity and heterozygosity promotes inbreeding depression and an accumulation of deleterious mutations, lowering the survival rates of inbred individuals (Boakes et al., 2007; Ficetola et al., 2011; Taylor, 2003). This can further reduce population size and increase the susceptibility of a population to stochastic processes (Ficetola et al., 2011; Frankham et al., 2010). Furthermore, the longer a population remains in captivity, the more liable it becomes to genetic adaptation through selective forces (Griffiths and Pavajeau, 2008). In the wild, genetic variation and selection allows evolutionary adaptation to environmental perturbations (Frankham et al., 2010; Taylor, 2003). However, genetic adaptation to captivity can occur in just a couple of generations (Williams and Hoffman, 2009) and potentially jeopardize restoration efforts by producing
an unsustainable population with a reduced ability to persist in its natural habitat on reintroduction to the wild (Ficetola et al., 2011; Williams and Hoffman, 2009).

Both inbreeding and adaptation to captivity can substantially be reduced by re-diversification through the introduction of individuals from other population fragments (Boakes et al., 2007; Frankham et al., 2009; Taylor, 2003). Traditionally, this would involve immigration of wild-caught individuals into captive populations or the transfer of captive individuals between breeding institutions (Taylor, 2003). However, most CBPs operate under limited space and resources and these management strategies are not always practical. In addition to disease transmission and welfare issues associated with the transfer of individuals between breeding populations are the logistical concerns of increasing captive population sizes. Furthermore, the removal of wild individuals for genetic augmentation of captive assurance colonies could detrimentally impact their free-living counterparts, particularly with regards to small or rare populations (Taylor, 2003).

Assisted reproductive technologies (ART) such as exogenous hormone treatment for the stimulation of gamete release, artificial fertilization (AF) for population augmentation, and gamete cryopreservation for genetic security, can be used to circumvent some of the genetic management limitations of a CBP. Increasing the carrying capacity and genetic diversity of a captive population without increasing its size can be achieved through ARTs. Previous research has shown that gamete release in male and female boreal toads can be induced by administration of exogenous hormones (Calatayud et al., 2015; Kouba and Vance, 2009; Langhorne et al., unpublished; Roth et al., 2010). The release of sperm, which is expressed in urine (spermic urine), can be achieved within 2 hours of exogenous hormone administration, and high sperm
concentrations can be maintained for up to 7 hours (Langhorne et al., unpublished). This minimally-invasive technique enables repeated sperm collections from individual males over several hours. Furthermore, motile spermatozoa from boreal toads has successfully been recovered from storage in cold-temperatures over several days, either through short-term storage at 4° C or through cryopreservation in liquid nitrogen (Langhorne et al., unpublished). Coupled with gamete collection techniques, the effective application of these cold storage methods offers a viable strategy for the collection and transportation of gametes from *ex-situ* breeding populations into CBPs, as well as the potential to transfer gametes between other wild population fragments. This field-oriented technique has successfully been applied to several fish species, with similar modes of reproduction, (Gorman, 2011).

The aim of this study was to present an alternative mechanism for introducing genetics into captive anuran populations in situations where wild cohorts remain. The potential for *in-situ* sperm collection from wild-caught boreal toads and the capacity to maintain viable sperm samples in cold storage was explored by applying lab-based gamete collection and cold storage techniques in the field. Furthermore, the feasibility of a genetic linkage strategy for *in-situ* and *ex-situ* boreal toad populations was investigated through evaluation of the functional capacity of spermatozoa, collected *in-situ*, and stored in cold-storage or cryopreserved, through AF with eggs from captive females.

Sperm concentration and motility were evaluated in a mobile workstation prior to cold storage or cryopreservation. The functional capacity of cold-stored and frozen-thawed sperm from wild males was assessed by AF using eggs collected from hormonally-induced captive female boreal toads housed at the NASRF facility. The
development of embryos from fertilization trials was then measured and followed through completion of metamorphosis.

**Methods**

**Animals**

**Wild-caught boreal toads**

Male toads (n=23) were located in the field over the course of three breeding seasons: May 2012 (n=6), May 2013 (n=12) and May 2014 (n=5). Gender and sexual maturity were identified by the presence of nuptial pads. Weight (g) and snout-vent-length (SVL) were recorded upon capture and males were placed in individual plastic holding tubs (18 cm L x 18 cm W x 10 cm H) containing 2 cm of water to ensure continuous urine production for the 5 h duration of sperm collections. Males were released back to their original capture location on completion of sperm collections. Male cohorts from the 2012 and 2014 breeding season were used for spermiation trials only. Male cohorts from the 2013 breeding season were used for spermiation and fertility trials with sperm stored at 4-6°C, or cryopreserved in liquid nitrogen.

**Study 1: In-situ spermiation induction and collection**

Figure 31 shows the various stages of sperm induction, collection and cryopreservation in the field. Prior to hormone stimulation, a urine sample (T₀) was obtained from each male by holding the individual above a petri dish until urination occurred, usually within one minute. Volume of T₀ urine was recorded and samples were maintained at 4°C until they could be evaluated for a spermiation response (determined by the presence or absence of sperm in the collected urine sample). Spermiation was
induced by intraperitoneal injection of 300 International Units (IU) of human chorionic gonadotropin hormone (hCG; #C1063; Sigma Aldrich, St Louis, Missouri, USA) and spermic urine was collected from each male into sterile 1.5ml Eppendorf tubes at 2, 3 and 5 h post-hormone administration (PA). The volume and collection time of each sample was recorded before samples were stored in a cooler at 4-6 °C until sperm quality parameters could be evaluated. The mobile workstation was an F250 Ford flatbed truck, where the interior was designated for sample processing using a microscope powered by a portable generator, and the exterior flatbed was converted into an area for sperm cryopreservation. Data collections were terminated at 5 h PA to allow departure from the field site within daylight hours.

**Spermic urine evaluation**

Spermic urine samples were examined under a 400x objective on an Olympus CX41 phase-contrast microscope. Samples were analyzed by placing a 10 µl aliquot of spermic urine onto a glass slide under a 400x objective on a phase-contrast microscope (Olympus CX41) and counting the first 100 sperm. Spermic urine variables measured included volume, and the percentage of spermatozoa exhibiting: Total Motility ([TM]; = flagellum movement); Forward Progressive Motility ([FPM] = forward progressive movement as a proportion of total count); and the Quality of Forward Progressive Motility ([FPM-Q] = a subjective scale from 0 (no movement) to 5 (very rapid forward movement). Sperm concentration was measured by inactivating sperm motility in a 1:10 dilution with phosphate-buffered saline (PBS) and using a Neubaeur haemocytometer to obtain average sperm concentration mL⁻¹.
Figure 31  Collection, processing and cryopreservation of boreal toad spermatozoa under field conditions

Clockwise from top left: Male toads located in the field were held in ventilated plastic containers; toad weight and SVL was recorded and a urine sample collected; 300 IU hCG was administered; spermic urine samples were processed in the mobile workstation; samples were stored in a cooler at 4° C until cryopreservation or artificial fertilization; cryopreservation workstation on the bed of the truck.

**Effect of short-term cold storage and cryopreservation on sperm quality parameters**

Spermic urine samples were maintained in a cooler at 4.9 ± 1.4 °C (recorded by Onset® HOBO® Data Logger; Onset Computer Corporation, Bourne, MA, USA) until sperm parameters could be evaluated in the mobile workstation (0.5-11.5 h post-collection [PC]), depending on proximity of field site to lab site, using the methodologies described above. Spermic urine samples were then either held in the cooler for cold-storage trials (Study 1; chilled sperm) or stored in liquid nitrogen for sperm cryopreservation trials (Study 2; frozen sperm).
Sperm short-term cold storage

Spermic urine samples collected from wild-caught male boreal toads ($n=9$) during the 2013 breeding season were selected for cold-storage if the following quality standards were met: $\geq 70\%$ total motility, $\geq 60\%$ FPM with progressive motility quality $\geq 3$, and sperm concentrations $>0.1 \times 10^6 \text{ mL}^{-1}$. A sub-set of males from Group 1 ($n=4$) and Group 2 ($n=5$), were collected on Day 1 and Day 2 of fieldwork, respectively. Chilled spermic urine samples were re-analyzed at 0 h, 48 h and 96 h PC (Group 1), and at 0 h 24 h and 72 h PC (Group 2) to quantify spermatozoa motility recovery (percent TM, percent FPM and FPM-Q). The difference in duration of cold storage between Group 1 and Group 2 spermic urine samples was a result of a 24 h period between the collection of Group 1 and Group 2 males. Sperm motility parameters of Group 1 males were analyzed on the day of collection in the mobile workstation (Day 1; 0 h PC) and stored at $4^\circ \text{C}$ until further motility analysis was conducted on return from the field at 48 h PC (Day 3). Group 2 males were collected on Day 2 of field work and sperm motility parameters were analyzed in the mobile workstation (0 h PC) and at 24 h PC (Day 3) on return from the field. The final motility analysis time-points of spermic urine samples was prior to artificial fertilization on Day 4, which was at 96 h PC and 72 h PC for Group 1 and Group 2 males, respectively.

Sperm cryopreservation

Spermic urine samples were selected for cryopreservation if the following quality standards were met: $\geq 70\%$ Total Motility, $\geq 30\%$ FPM with Forward Progressive Motility Quality $\geq 3$, and sperm concentrations $>1.0 \times 10^6 \text{ mL}^{-1}$. Spermic urine aliquots (100 µl) from each male were extended 1:1 in chilled cryoprotectant (CPA) solution containing a final concentration of 10% (v/v) dimethyl formamide (cat# D4551; Sigma-
Aldrich) in a 0.5 M trehalose (cat# T5251; Sigma-Aldrich) diluent. Spermic urine was frozen using a two-step slow-rate freezing protocol. Cryosuspensions were held at 4° C for 10 min before loading into 0.25 mL plastic freezing straws (Minitube International®; Germany). Following this, straws were placed in liquid nitrogen vapor on a platform 10 cm above the level of liquid nitrogen in a Styrofoam freezing box for a further 10 min, before plunging into liquid nitrogen and storing in a dry shipper at -90 °C. Straws were rapidly thawed by immersion in a 40 °C water bath until all visible ice crystals had melted (~ 5 s). Thawed sperm suspensions were expelled into a petri dish and activated by a 1:10 dilution with aged tap water and sperm motility parameters (TM, FPM and FPM-Q) were recorded.

**Study 3: Fertilization capacity of chilled and frozen-thawed sperm**

Artificial fertilization trials were performed during the 2013 breeding season. Captive female boreal toads (n=2) at NASRF were removed from hibernation and acclimated at room temperature for a period of 3 days. Throughout the duration of the trial, females were held in individual plastic holding tubs (18 cm L x 18 cm W x 10 H cm), with access to water, until egg deposition occurred.

**Ex-situ oocyte recovery artificial fertilization (AF)**

Stimulation of ovulation and spawning was achieved using the following exogenous hormone protocol: two hCG priming doses (3.7IU g⁻¹ Body Weight [BW]) 4 days apart, followed 24 h later by a combined ovulatory dose of hCG (3.7 IU g⁻¹ BW) and gonadotropin-releasing hormone analog (0.4 µg g⁻¹) (GnRHα; [des-Gly¹⁰, D-Ala⁶]-LH-RH-ethylamide acetate]cat# L4513; Sigma Aldrich, St Louis, Missouri, USA). On
initiation of egg deposition, females were gently squeezed to facilitate egg expression into a 150 mm petri dishes. For fertilization trials using fresh sperm, a control male ($n=1$) was held in an individual plastic holding tub (18 cm L x 18 cm W x 10 cm H), containing 2 cm water, and administered 300 IU hCG. Sperm was collected at 2, 3 and 5 h PA to mimic the wild sperm time-point collections and analyzed as described above.

Twenty fertilization dishes were used for each female/male fertilization pair (2 x chilled sperm dishes, 2 x frozen-thawed sperm dishes per males + 2 x fresh control dishes + 2 x parthenogenic control dishes). Fresh oocytes (123 ± 4 oocytes per dish; mean ± SEM for 36 dishes) were deposited into 150 mm petri dishes and 100 µl sperm was pipetted onto the egg mass for a dry fertilization period of 5 min before flooding the dish with aged tap water. Fertilization rates were assessed by counting 2 - 4 cell embryos, under a 10x objective on a dual-power stereo microscope (Omano, OM4713) 4 - 6 h post-fertilization. Any non-fertilized or dead embryos were removed a few days later and the remaining viable embryos were monitored through metamorphosis.

**Tadpoles**

On hatching (Gosner stage 20), all tadpoles were transferred from 150 mm petri dishes into larger plastic tubs (28 cm L x 20 cm W x 28 cm H) and fed ground tropical fish flakes (Tetra®, United Pet Group, Blacksburg, VA, USA) 1 -2 times per day and algae pellets (Tetra®) *ad libitum*. Partial water changes (30- 50%) were performed every 48 h. Upon emergence of front limbs (Gosner stage 42), tadpoles were moved into smaller tubs (50 cm L x 25 cm W x 35 cm H) containing 1 L aged tap water. Each tub was placed at a 20° degree angle such that water pooled in only one half of the tank to facilitate exit from
the water on full limb emergence and the completion of metamorphosis (characterized by complete tail regression; Gosner stage 46).

**Statistical analysis**

Assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk and Levene’s test, respectively. A one-way analysis of variance (ANOVA) was performed to compare toad body measurements (weight and SVL), sperm quality parameters (concentration, TM, FPM and FPM-Q) across the three seasons and sperm quality parameters of short term cold stored sperm at each analysis time-point (0, 24, 48, 72 and 96 h PC). Sperm motility data was assessed using paired student t- tests for comparison of pre-freeze and post-thaw spermatozoa motility parameters from each male. All percentage data were arcsine transformed using the transformation \( \sin^{-1}(\sqrt{x}) \) before further analysis. All data are expressed as means ± SEM. Significance was established at \( P \leq 0.05 \). All statistical analysis was performed in SAS Version 9.4 (Cary, North Carolina, USA).

**Results**

**Study 1: In-situ spermiation induction and collection**

Table 9 summarizes weight and SVL measurements and sperm quality parameters (concentration, TM, FPM and FPM-Q) as means of the total number of toads in each separate breeding season [2012, 2013 and 2014]. Body characteristics (weight and SVL) were significantly lower (one-way ANOVA; \( P < 0.05 \)) in males collected during the 2012 breeding season than males collected during 2013 and 2014. There was no significant difference (one-way ANOVA; \( P > 0.05 \)) in mean sperm concentration between male
groups from each breeding season however, males from 2014 produced significantly higher volumes of spermic urine (one-way ANOVA; $P<0.05$; Table 9).

Sperm TM and FPM were significantly lower (one-way ANOVA; $P<0.05$) in spermic urine samples from males in 2012 (60.8 ± 6.0% and 32.5 ± 6.7%, respectively) than sperm from males in 2013 (89.5 ± 2.6% and 71.7 ± 4.1%, respectively) and 2014 (85.8 ± 3.5% and 63.0 ± 6.1%, respectively; Table 9). The quality of FPM (FPM-Q) ranged from 3-4 across all three breeding seasons with no significant difference between male cohorts in each breeding season (Table 9).

The following data refer to the accumulated values from each breeding season, unless specified otherwise. The mean weight and SVL of wild-caught male toads ($n = 23$) was 42.3 ± 2.1 g and 71.6 ± 1.3 mm, respectively. Urine analysis prior to hormone injection indicated that sperm was absent from all but one male. As a result, the individual male was excluded from any subsequent data analysis.
Table 9  Mean weight, SVL and sperm quality parameters in spermic urine samples of wild-caught male boreal toads from each breeding season

<table>
<thead>
<tr>
<th></th>
<th>2012</th>
<th>2013</th>
<th>2014</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>33.3 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.7 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.8 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Snout-vent length (mm)</td>
<td>65.7 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.6 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74.0 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spermic urine volume (µl)</td>
<td>732 ± 60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>639 ± 21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1105 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sperm concentration (x10&lt;sup&gt;6&lt;/sup&gt; mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>1.5 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.1 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>60.8 ± 6.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89.5 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85.8 ± 3.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sperm forward progressive motility (%)</td>
<td>32.5 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.7 ± 4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63.0 ± 6.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sperm forward progressive quality (0-5)</td>
<td>3.2 ± 0.4</td>
<td>4.1 ± 0.2</td>
<td>3.8 ± 0.2</td>
</tr>
</tbody>
</table>

Mean weight and SVL were significantly higher (P<0.05) in males from the 2013 and 2014 breeding season. Spermic urine volume was significantly higher in males collected in 2014 (P<0.05) however, sperm concentration did not differ significantly (P>0.05) between male groups from each breeding season (2012, 2013 or 2014). Total Motility [TM] and Forward Progressive Motility [FPM] were significantly lower (P<0.05) in sperm collected during the 2012 breeding season. Forward Progressive Motility Quality [FPM-Q] ranged from 3-4 across each breeding season. Body parameter values (Weight and SVL) are expressed as means of the total number of males from each separate breeding season Data collected from spermic urine analysis (volume, concentration, TM, FPM and FPM-Q) are expressed as means of the total number of males responding at each time-point post-hormone administration ± SEM.

Administration of exogenous hCG treatment induced spermiation in 96% (22/23) of male toads, only one injected male did not respond to hormone treatment at all three time-points (2 h, 3h and 5 h). (Figure 32.A. and B.). Sperm was released into urine within 2 h PA in 91% of males, and sperm continued to be released at each subsequent collection time-point (2 h, 3h and 5 h PA; Figure 32.A.). At 2 h PA the mean concentration of sperm was 3.25 ± 0.7 x 10<sup>6</sup> mL<sup>-1</sup>, with peak production occurring at 3 h PA in 96% of male toads (3.5 ± 0.3 x 10<sup>6</sup> mL<sup>-1</sup>; Figure 32.B.). At the final collection time-point (5 h PA) mean sperm concentration had declined to 2.5 ± 0.6 x 10<sup>6</sup> mL<sup>-1</sup> in 87% of hormone-treated male toads (Figure 32.B.). Mean sperm Total Motility (TM) was
consistently high between 2 - 5 h PA (79.0 ± 4.2% - 81.9 ± 4.2%; Figure 33.A.). Forward Progressive Motility (FPM) analysis of sperm showed an increase from 52.1 ± 6.0% at 2 h PA to 63.3 ± 6.9% by 5 h PA and the quality of FPM (FPM-Q) maintained a consistently high ranking of 4 (out of a maximum of 5) across all time-points PA (Figure 33.A and B.).

Figure 32  Spermiation response (A) and sperm concentration (B) of wild-caught male *A. b. boreas* over 5 h following exogenous hormone administration of hCG

Urine was collected from wild-caught male boreal toads (*n*=23) at 3 time-points (2, 3 and 5 h) post-hormone administration [PA] of 300 IU hCG. A spermiation response was initiated within in 91% of male toads within 2 h PA. Peak sperm production occurred at 3 h PA when 96% of hormone-treated toads produced a mean sperm concentration 3.5 x ± 0.3 x 10^6 mL^{-1}. Responding males were classified as individuals producing spermic urine at a given collection time-point PA. Values are expressed as untransformed percentages (A) or means ± SEM (B) of the total number of hormone-treated male toads spermiating at each collection time-point ± SEM.
Figure 33  

Mean TM and FPM (A), and FPM-Q (B) of spermatozoa from wild-caught male *A. b. boreas* over 5 h following exogenous hormone administration of hCG.

Total Motility [TM] and Forward Progressive Motility [FPM] (A) and Forward Progressive Motility Quality [FPM-Q] (B) of sperm collected from wild-caught male boreal toads (*n* = 23) at 3 time-points (2, 3 and 5 h) post-hormone administration [PA] of 300 IU hCG. Sperm TM and FPM ranged from 79.0 % ± 4.2 - 81.9 % ± 4.2% and 52.1 ± 6.0% - 63.3 ± 6.9%, respectively, across the three collection time-points. Sperm FPM-Q maintained a mean quality rating of 4 across the three collection time-points PA. Data shown are untransformed percentages (A) or means ± SEM (B) of the total number of hormone-treated male toads spermiating at each collection time-point ± SEM.

Figure 34 details the field site locations throughout the three breeding seasons.

Field Site 1 (South Cottonwood West; 2012) and Field Site 3 (Four Mile; 2014) were in relatively remote locations with respect to the mobile workstation. As such, sperm analysis occurred at 7- 11.5 h and 6- 9 h PC in 2012 and 2014, respectively. Field Site 2 (South Cottonwood Creek; 2013) was located within 5 min of the mobile workstation and sperm motility was analyzed within 0.5– 6 h PC.
Figure 34  Field locations of in-situ sperm collections during breeding seasons 2012, 2013 and 2014

Left to right:  Field Site 1: South Cottonwood West (Altitude: 10 000 ft; n =7 males collected; Season: 2012); Field Site 2: South Cottonwood Creek (Altitude 10 446 ft; n=12 males collected; Season: 2013); Field Site 3: Four Mile (Altitude: 10 200 ft; n =5 males collected; Season: 2014)

**Study 2: Effect of short-term cold storage and cryopreservation on sperm quality parameters**

**Sperm short-term cold storage**

Short-term cold storage techniques were employed in the field to explore the potential for transportation of viable sperm samples, collected from in-situ males into captive breeding facilities. This study quantified the motility capacity of short-term cold stored sperm samples by analysis of TM, FPM and FPM-Q at 0, 48 and 96 h PC (Group 1 males; n=4; Figure 35.A.) and at 0 h, 24 h and 72 h PC (Group 2 males; n =5; Figure 35.B.).

Following 48 h in cold storage, there was a 23% and 31% reduction in sperm TM and FPM, relative to initial motility analysis (0 h), respectively (Figure 35.A and B.). Sperm quality maintained the initial quality rank of 4 (Figure 35.C.). Motility analysis in Group 1 after a further 48 h in cold storage (96 h PC) showed continued declines in sperm TM and FPM by an additional 30% and 23%, respectively (Figure 35.A and B.), with FPM-Q reduced to a quality rank of 3 (Figure 35.C.). Overall, after 96 h in cold
storage, sperm motility parameters in Group 1 male spermic urine samples showed a significant (one-way ANOVA; \( P<0.05 \)) total loss of 46% TM from initial analysis (0 h; TM = 97 ± 1.1%), resulting in a final mean TM of 44.3 ± 12.11% (Figure 35 A.). The proportion of sperm exhibiting forward motility was significantly reduced (one-way ANOVA; \( P<0.05 \)) compared to sperm FPM at initial analysis (0 h; 81 ± 5.2%), resulting in a final FPM of 26.3 ± 10.3%, and a reduction in FPM-Q by one rank (4 to 3) at 96 h PC (Figure 35. B. and C.).

Similar sperm motility patterns were exhibited in the spermic urine samples of Group 2 males. After 24 h in cold storage sperm TM and FPM reduced by 19% and 18% from initial motility analysis (0 h), respectively (Figure 35.A.and B.), with FPM-Q maintaining the initial quality rank of 4 (Figure 35.C.). Motility analysis in Group 2, after a further 48 h in cold storage (72 h PC) showed continued declines in spermatozoa TM and FPM by a further 30% and 38%, respectively (Figure 35.A.and B.), and a 50% reduction in FPM-Q to a rank quality of 2 (Figure 35.C.). Overall, after 72 h in cold storage, spermatozoa motility parameters in Group 2 male spermic urine samples showed a significant (one-way ANOVA; \( P<0.05 \)) total loss of 36% TM from initial analysis (0 h; TM=88.2% ± 3.4), resulting in a final mean TM of 31.5% ± 15.4 (Figure 35.A.). Sperm FPM was significantly (one-way ANOVA; \( P<0.05 \)) reduced by 42% from initial analysis (0 h; 66.8 ± 6.5%) resulting in a mean FPM of 29.0 ± 16.7%, and a reduction in FPM-Q by two quality ranks (4 to 2) at 72 h PC (Figure 35. B. and C.).

In summary, boreal toad spermatozoa held in cold storage for up to 96 h maintained between 30% and 45% of total spermatozoa motility, of which between 25% and 29% retained forward progressive motility with a quality rank of 2-3.
Spermic urine samples \((n=9)\) from wild-caught male boreal toads during the 2013 breeding season were maintained at 4\(^\circ\) C for a storage period of 96 h (Group 1) or 72 h (Group 2) post-collection (PC). Motility parameters (TM, FPM and FPM-Q) of spermic urine from Group 1 males \((n=4)\) were analyzed at 0 h, 48 h and 96 h PC and from Group 2 males \((n=5)\) at 0 h, 24 h and 96 h PC. In both Group 1 and Group 2, sperm motility parameters declined significantly \((P<0.05)\) by 96 h and 72 h, respectively. Data shown are untransformed means of the total number of spermic urine samples exhibiting motility at each analysis time-point PC ± SEM.

Figure 35  TM (A), FPM (B) and FPM-Q (C) of sperm from wild-caught male *A. b. boreas* after cold-storage at 4\(^\circ\)C for up to 96 h post collection.
**Sperm cryopreservation**

The ability to maintain sperm samples in cold storage for a number of days affords many practical benefits to a captive breeding operation. However, the long-term storage of sperm through cryopreservation confers additional advantages to a CBP in terms of genetic management and the security of genetic diversity. In this study, a sub-set of spermic urine samples from males \( n=4 \) used in the short-term cold storage trials was selected for sperm cryopreservation in order to remove any inter-male variation and strengthen the comparison between fertilization capacity of chilled spermatozoa and frozen-thawed spermatozoa. Therefore, each male served as its own fresh control for the effects of cryopreservation on post-thaw sperm motility recovery and fertilization capacity. The sperm cryopreservation methodology presented in this study is straightforward to apply in a field setting, and utilizes relatively basic equipment, comprising a cooler maintained at 4-6° C for maintaining spermic urine samples prior to cryopreservation, a simple Styrofoam box for holding liquid nitrogen for cryopreservation, and a dry shipper for the temporary storage and transportation of frozen sperm samples.

Males \( n=4 \) were selected for cryopreservation depending on the volume and quality of the spermic urine samples collected. Cryopreservation techniques were employed *in-situ* and straws were thawed on return to NASRF between 72 – 96 h post-collection. Post-thaw sperm TM from each male ranged between 22.2 ± 1.6% - 53.9 ± 3.4%, of which 4.9 ± 2.1% – 23.9 ± 2.0% exhibited FPM with a mean quality of 1.4 ± 0.3 – 3.0 ± 0.2 (Table 10). Post-thaw sperm motility parameters [TM, FPM and FPM-Q] were significantly lower \( P<0.05 \) than initial pre-freeze sperm motility for all 4 males.
On average, frozen-thawed sperm recovered between 31% - 57.3% and 13% – 28.5% of initial TM and FPM, respectively (Post-thaw Motility/Initial Motility x 100). The quality of frozen-thawed sperm was significantly reduced to 1.4 ± 0.3- 3.0 ± 0.2 (Table 10).

**Table 10**  Sperm motility parameters from wild-caught male *A. b. boreas* before (pre-freeze) and after (post-thaw) cryopreservation

<table>
<thead>
<tr>
<th>Male No. straws</th>
<th>Pre-freeze MOT (%)</th>
<th>Post-thaw MOT (%)</th>
<th>Pre-freeze FPM (%)</th>
<th>Post-thaw FPM (%)</th>
<th>Pre-freeze FPM-Q (0-5)</th>
<th>Post-thaw FPM-Q (0-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>94</td>
<td>53.9 ± 3.4</td>
<td>84</td>
<td>23.9 ± 2.0</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>87</td>
<td>42.4 ± 3.2</td>
<td>72</td>
<td>11.8 ± 3.5</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>89</td>
<td>34.8 ± 3.8</td>
<td>73</td>
<td>10.8 ± 2.2</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>70</td>
<td>22.2 ± 1.6</td>
<td>37</td>
<td>4.9 ± 2.1</td>
<td>3</td>
</tr>
</tbody>
</table>

Spermic urine from hormonally-stimulated wild-caught boreal toad males (*n*=4) was cryopreserved in a dimethyl-formamide based cryoprotectant solution. Post-thaw sperm total motility [MOT] and forward progressive motility [FPM] ranged between 22.2 ± 1.6% -53.9 ± 3.4% and 4.9 ± 2.1% -23.9 ± 2.0%, respectively. Post-thaw sperm quality [FPM-Q] ranged between 1.4 ± 0.3 and 3.0 ± 0.2. Values are expressed as means of the total number of straws (*n*= 9- 15) thawed for each male ± SEM.

**Study 3: Fertilization capacity of chilled and frozen-thawed sperm**

The functional capacity of sperm maintained either in cold-storage at 4 °C (chilled) or cryopreserved in liquid nitrogen (frozen) was evaluated through artificial fertilization (AF) using eggs from captive female toads (*n*=2). Females initiated spawning 12 h following administration of an ovulatory hormone dose (72 – 96 h post-sperm...
collection in the field for Group 2 and Group 1, respectively). The following data is expressed as means of the total number of fertilization dishes for each male/female pairing. Parthenogenic control dishes exhibited $11.6 \pm 4.8\%$ cleavage but no further developmental stages were observed. Fertilization rates of the freshly collected sperm from the control male ($n=1$) were significantly higher ($P<0.05$) than fertilization rates of frozen-thawed sperm ($57.8 \pm 20.9\%$ and $2.8 \pm 1.4\%$, respectively; Table 11). However, there was no significant difference between the fertilization rates of fresh control and chilled spermic urine samples ($27.5 \pm 11.3\%; P>0.05$; Table 11). A total of 493 and 92 tadpoles completed metamorphosis from chilled sperm and frozen-thawed sperm AF, respectively (Table 11).

Table 11 Percentage of embryos at cleavage and tadpole stage, and the total number of tadpoles completing metamorphosis in *A. b. boreas* artificial fertilization trials using chilled and frozen-thawed sperm

<table>
<thead>
<tr>
<th>Male</th>
<th>Cleavage rate (%)</th>
<th>Tadpole (%)</th>
<th>No. metamorphosed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chilled Frozen</td>
<td>Chilled Frozen</td>
<td>Chilled Frozen</td>
</tr>
<tr>
<td>1</td>
<td>22 19</td>
<td>12 7</td>
<td>65 76</td>
</tr>
<tr>
<td>2</td>
<td>54 2</td>
<td>58 2</td>
<td>277 10</td>
</tr>
<tr>
<td>3</td>
<td>34 1</td>
<td>30 1</td>
<td>146 5</td>
</tr>
<tr>
<td>4</td>
<td>3 1</td>
<td>1 1</td>
<td>5 1</td>
</tr>
<tr>
<td>Mean (%)</td>
<td>27.5 ± 11.3 5.75 ± 4.4</td>
<td>25.25 ± 12.4 2.75 ± 1.4</td>
<td>- -</td>
</tr>
</tbody>
</table>

Sperm from wild-caught boreal toads ($n=4$) was either cold-stored (chilled) or cryopreserved (frozen) and used for artificial fertilization with eggs expressed from captive NASRF females ($n=2$). Fertilization and tadpole rates are expressed as percentages of the total number of eggs in fertilization dishes per male/female breeding pair. Metamorphosed totals represent the combined number of tadpoles that completed metamorphosis per male/female breeding pair. Values are expressed as means ± SEM.
Discussion

In the present study a genetic linkage technique was developed for the introduction of genetic variation into a captive anuran colony by quantifying spermiation response to exogenous hormone administration in wild-caught *A. b. boreas* under field conditions. In addition, the functional competence of sperm collected *in-situ* was explored through evaluation of the effects of both long and short-term cold-storage techniques on sperm motility and fertilization capacity.

In total, 24 male boreal toads were located at three different breeding sites across three breeding seasons (2012, 2013 and 2014). Spermic urine production was induced within 2 h post-hormone administration [PA] in 96% of male toads with a mean concentration of $3.5 \times 10^6$ mL$^{-1}$. The concentration remained high across all three collection time-points (2, 3 and 5 h PA) in conjunction with a high motility rate, corresponding to the spermiation profile reported in captive boreal toads (Langhorne *et al.*, unpublished). In the present study, spermic urine was only collected up to 5 h PA, but the collection time could be extended as boreal toads have been shown to produce sperm up to 24 h PA (Kouba and Vance, 2009; Langhorne *et al.*, unpublished). For the purpose of cryobanking *in-situ* genetics for future breeding, it would be recommended to continue sample collections at least up to 12 h PA in order to maximize the opportunity to secure a sufficient supply of frozen samples.

Sperm samples collected from the 2012 male cohort had significantly lower sperm motility parameters than male groups from 2013 and 2014, however, sperm production was not significantly different between the three breeding cohorts. The 2012 breeding site (South Cottonwood West; Figure 33) was located relatively remotely and as
such, the spermic urine samples were maintained in cold storage for a longer period prior to processing the sample (7 h –11 h post-collection [PC]). The proximity of field site location, and corresponding length of time in cold storage, might explain the significantly reduced sperm motility parameters from 2012 male spermic urine samples. However, Field Site 3 (Four Mile; Figure 34) was also remotely located, and sperm analysis occurred between 6-9 h PC, suggesting that the duration in cold storage did not account for the discrepancy in sperm quality between these two breeding seasons. A more likely explanation is that the cooler used for cold storage in 2012 was insufficiently insulated and may not have maintained the spermic urine samples at an adequate temperature. The cold storage unit introduced in 2013, with the addition of a temperature monitoring device, appeared to maintain significantly higher quality spermic urine samples.

The cryopreservation technique outlined in the present study required relatively inexpensive equipment and has been used to successfully cryopreserve sperm from two other Bufonid and one Ranid species (Anaxyrus fowleri, Peltophryne lemur and Lithobates sevosa; Langhorne et al., unpublished). However, studies in fish, have shown poor reproducibility in freezing protocols using a Styrofoam freezing box at the field level, due to species-specific differences in freezing rates (Gilmour, 2005). Future application of this cryopreservation protocol for sperm collections would perhaps benefit from an improved portable freezing system. Magyary et al. (1995) described a self-sustained portable kit for in-situ gamete collection in fish in which cooling rates could be altered through an adjustable straw platform, and different sperm packaging materials (i.e. straws, cryovials) could be accommodated. The development of a standardized field
kit would be beneficial for future *in-situ* sperm collections to extend the potential application of these methods to a wider range of anuran species.

Cryopreservation of sperm samples in the present study was made possible by the proximity of the field site to the mobile workstation. However, this might not always be a practical option, particularly in very remote locations only accessible by foot. Therefore, further investigation into alternative freezing methods, such as on dry ice or in the vapor of a fully-charged dry shipper, would be worthwhile. Alternatively, the cold-storage study described herein demonstrates the ability to store sperm for several days and retain fertilization capacity, providing a means to collect sperm remotely and cryopreserve samples after a number of days. However, this method might substantially reduce post-thaw motility of frozen sperm. Browne *et al.* (2002) reported moderate freeze-thaw recovery of *Bufo marinus* sperm held at 0° C for 6 days before cryopreservation and Fowler’s toad spermic held in cold-storage at 4° C for 48 h prior to cryopreservation exhibited poor post-thaw motility recovery [pers. observ.]. In the anurans the sperm is immotile in the testis until it is released into a hypotonic environment, therefore spermic urine samples maintained in cold-storage are stored in a naturally-activated state (Duellman and Trueb, 1994; Kouba *et al.*, 2003). The extension of spermic urine in a high osmolality diluent, that deactivates motility, might preserve sperm motility for longer in cold-storage. Browne *et al.* (2001) deactivated *B. marinus* sperm samples in a concentrated dilution prior to cold storage and demonstrated that fertilization rates were significantly improved and fertilization capacity was maintained up to 10 days. It would be worthwhile exploring potential concentrated solutions for increasing the longevity of boreal toad sperm.
The long-term maintenance of genetic diversity in captivity can be challenging and there is often no way to know when, if ever, introductions to the wild will take place (Frankham et al., 2010; Loeffler, 2001). Therefore, maintaining the capacity for evolutionary response to a dynamic environment, is crucial for maximizing the success of reintroductions, particularly for species held in captivity for a considerable length of time (Moritz, 2013). Sperm collection and cryopreservation of wild-caught male boreal toads offers a potential means to minimize genetic differentiation between captive and wild populations by restoring valuable genetics (Frankham et al., 2010). Therefore, efforts should be made to cryopreserve sperm from both in-situ and ex-situ populations in order to avoid genetic adaptation to captivity, as well as natural and artificial selection over time (Williams and Hoffman, 2009). The captive NASRF boreal toad colony could substantially benefit from the opportunity to increase genetic diversity through the introduction of gametes from corresponding in-situ regional population segments.

In the wild, migration between breeding sites is a natural re-diversification process, however in fragmented populations the reduced ability of toads to migrate between breeding ponds limits the gene pool (Frankham et al., 2010). A number of boreal toad breeding sites are no longer occupied, increasing the distance between wild cohorts, and reducing the opportunities for immigration (Loeffler, 2001). Immigration requires as many as 10 migrants per generation to be effective and, in the case of the boreal toad, there are not enough wild individuals available (Lacy, 1987). Sperm collections from wild-caught boreal toad males could provide a useful means to facilitate gene flow between fragmented wild populations. Therefore, the transfer of genetics between
fragmented wild populations through *in-situ* sperm freezing, egg expression, and artificial fertilization in the field warrants exploration (Lacy, 1987).

As with any novel technique, potential drawbacks should be weighed up alongside the obvious returns. Disease transmission is always a risk for captive breeding programs both within and between facilities. It remains unknown whether or not amphibian diseases can be vertically transmitted through sperm and if disease is detected then sperm cannot be used in frogs to be reared for release. Duffus *et al.* (2008) reported a ranavirus positive wood frog (*Rana sylvatica*) larva that hatched from an egg fertilized by a ranavirus-negative female and a ranavirus-positive male, suggesting a vertical transmission mechanism. The reproductive tract of the amphibians share common anatomy with the urinary and gastrointestinal tracts, therefore there is a chance for sperm to contact potentially infected tissue (Duffus *et al.*, 2008). Spermatozoa could potentially be exposed to a virus during spermatogenesis, as is the speculated mechanism for iridovirus-associated tiger salamander (*Ambystoma maculatum*) mortality, proposed by Docherty *et al.* (2003), following isolation of the virus within the testis. Therefore, a risk assessment of the possibility of vertical disease transmission would be necessary, with consideration extending to the development of screening techniques and sperm packaging equipment. For example, the use of freezing straws might mandate heat-sealing of straws which have a lower potential for contamination compared to a vinyl plastic plug (Morris, 2007).

A further potential issue with regards biosecurity is liquid-nitrogen mediated disease transmission. The storage of multiple sperm samples from multiple geographical locations could potentially contaminate the liquid nitrogen within biobank with...
pathogenic agents that could, in theory, be transmitted to other stored samples. However, over the last 30 years, there have been no reports of disease transmission related to gamete storage in liquid nitrogen, in humans or wildlife (Kuwayama, 2012). The theoretical risk does exist though, and should be minimized by adopting a number of practical measures, such as ensuring that samples are appropriately sealed for storage (i.e. closed straws), and liquid nitrogen used in the cooling and warming process is factory-sourced (Kuwayama, 2012)

To the best of the author’s knowledge the present study represents the first successful application of assisted reproductive technologies for the recovery and storage of spermic urine under field conditions. Furthermore, the successful production of offspring validates this genetic linkage technique as a viable option for increasing the genetic diversity of captive *A. b. boreas* populations through the seasonal integration of gametes from their wild counterparts. The cold-storage and freezing techniques presented in this study could have applications to a number of other threatened or declining frog and toad species in the wild. However, a cooperative relationship with field biologists will be key to the implementation of these techniques on a wider scale. The boreal toad is not a listed species and therefore, not tightly regulated. However, this field technique may be more challenging to execute with regards federally listed species, where acquisition of the requisite permits could limit its application.

**Acknowledgements**

This study was supported an Institute of Museum and Library Services National Leadership Grant (LG-25-09-0064-09) and a Memphis Zoo Conservation Action Network Award. The authors would like to thank the Mississippi Agriculture and
Forestry Extension Service (MAFES) and the Colorado Division of Wildlife, in particular Harry Crocket, Ted Smith, Scott Schneider, Bradley Lambert, Tom Mix and all the staff at the Native Aquatic Species Restoration Facility for accommodating our research and providing assistance in the field. We are also grateful to Michael Robinson, Christy Steadman and Andrew House for their help with the husbandry and care of the MSU boreal toad colony.
References


IUCN Red list of Threatened Species. Version 2014.3 (IUCN, 2015; www.iucnredlist.org)


Loeffler, C. (ed.) et al. (2001) Conservation plan and agreement for the management and recovery of the southern Rocky Mountain population of the boreal toad (Bufo boreas boreas). Boreal Toad Recovery Team. pp. 76+ appendices


CHAPTER VIII

CONCLUDING REMARKS

We are in a race against extinction; and we are losing. Therefore, we must urgently pursue the development of assisted reproductive technologies (ARTs) for the captive propagation of amphibian species driven to the brink of extinction, and those following closely behind. These studies set out to develop and refine ARTs for three North American anuran species of conservation concern. The valuable information gained herein will supplement our growing foundation of knowledge to support the successful and efficient captive management of a taxon with such complex reproductive adaptations so as to provide the ultimate challenge for amphibian reproductive biologists.

With an overwhelming number of species in need of protection, we must endeavor to save the frogs with all the tools at our disposal. It is essential that ARTs are promoted as valuable intermediary measures, complimentary to in-situ conservation efforts. This can only be accomplished by adopting a multidisciplinary approach, requiring the integration of both in-situ- and ex-situ-based research. Furthermore, we must forge a compelling narrative to educate and engage the wider community by championing the frogs and promoting their crucial contribution to the health of the planet and in turn, humanity.

I feel hugely privileged to have been afforded the opportunity to embark on the research objectives presented in this manuscript and to contribute my findings to the
global amphibian rescue mission. The sheer scale of the conservation challenge ahead can, at times, feel insurmountable. However, despite highlighting the profoundly disturbing irony of a simultaneous global species loss, the discovery of new amphibian species is enormously motivating; because perhaps more upsetting than the irretrievable loss of a species before our eyes, is the loss of one that has never been seen.