ABSTRACT

Title of Document:	STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE PANAMANIAN GOLDEN FROG (*ATELOPUS ZETEKI*) SPERMATOZOA – IMPACT OF MEDIUM OSMOLALITY AND CRYOPRESERVATION ON MOTILITY AND CELL VIABILITY
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The Panamanian Golden Frog (*Atelopus zeteki*, Anura: Bufonidae), an endemic species from Panama, is critically endangered and believed to be extinct from the wild. Infectious disease, habitat destruction and pet trade are among the major causes of the populations decline. Conservation initiatives in Panama and the US have established captive breeding programs for the species but there is still much to be learned about its reproductive physiology to manage and sustain populations using Assisted Reproductive Technologies (ARTs).

The main objectives of this dissertation were: 1) characterize the general sperm parameters and assess the effect of hormonal stimulation on motility, forward progressive motility, DNA integrity, sperm morphology and seasonality; 2) identify the effect of extracellular conditions, mainly dilution and temperature, on motility, forward progressive motility, duration of motility, morphology and DNA integrity; and 3)

evaluate the effect of cryoprotectants on motility, forward progressive motility and sperm DNA integrity before and after freezing.

Results demonstrated that: 1) *A. zeteki* sperm morphology is similar to that described in other Bufonids; the species successfully produces high quality spermatozoa when stimulated with intraperitoneal injection of Amphiplex, GnRH and hCG; hormonal stimulation is not detrimental to the motility parameters, cell morphology or DNA integrity; and, there is no seasonal effect on the response to the hormonal stimulation. These results indicate that the use of hormone treatments can be included in captive breeding programs to safely collect good quality sperm; 2) dilution of spermic urine in water highly reduces sperm motility, forward progressive motility, DNA and morphological integrity while storage of spermic urine at 4 °C preserves sperm quality for at least 46 min after collection; and 3) some recovery of viable *A. zeteki* spermatozoa after cryopreservation can be achieved by equilibrating the ARS-diluted samples for 5 min at 4 °C in CPA3-REY, using step-wise cooling before plunging the samples in LN_2. Collective results offer missing information on the reproductive biology of the male *A. zeteki* and lead to the application of ARTs for the captive management of this charismatic but critically endangered species.

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Preface

Amphibian populations around the world are drastically declining with entire species going extinct. Habitat destruction and infectious diseases are the main causes of the current amphibian extinction crisis, in which 122 species have gone extinct since the 1980's. The Smithsonian Conservation Biology Institute, through its Amphibian Conservation Program, in collaboration with the Smithsonian Tropical Research Institute (STRI), the Africam Safari, Defenders of Wildlife, the Cheyenne Mountain Zoo, the Houston Zoo, Summit Municipal Park and Zoo New England, with additional collaboration of El Valle Amphibian Conservation Center (EVACC) have responded to the amphibian crisis with the creation of conservation programs in Panama and the US. In April 2015, the Panama Amphibian Rescue and Conservation Project opened its doors as the largest amphibian rescue and research facility of the world. Research in fields like amphibian reproduction, skin microbiomes, genomics, amphibian and veterinary care are the main focus of this great initiative and is contributing to the conservation of recently discovered and already endangered species.

Dedication

This dissertation is dedicated to Clara Nieto Della Togna† and Reinaldo Della Togna, my beloved parents, for their unconditional support and encouragement, their inspirational life example and for having always believed in me; to my husband Rafael Milanes and my siblings Marisel Della Togna, Clara Della Togna and Reinaldo Della Togna, for their absolute faith in me and support, and to my friends Ana Elena Restrepo, Ines Ruiz and Wilfredo Bermudez, for always being there.

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The amphibian extinction crisis and the genus *Atelopus*

Although amphibian declines were noticed as early as the 1950's, it was not until the first World Congress of Herpetology in1989, that scientists from around the world realized the extent of the problem, where these were not isolated "natural" declines but a major global crisis. Furthermore, the meeting served as the platform to evaluate the global scale of amphibian declines, marking a turning point in the amphibian conservation world [1,2]. As a result, the IUCN's Species Survival Commission formed the Declining Amphibian Population Task Force (DAPTF) in 1990, with the objective of assessing the complexity of the crisis, identify species and populations in need of immediate attention and to understand the cause(s) of the declines [1,3,4]. The work done by the DAPTF in approximately 90 countries resulted in a great amount of research and publications that led to the launching of the IUCN's Global Amphibian Assessment (GAA) in 2004 [1,2]. The GAA's main goal was to assess the conservation status and imminent threats for all described amphibian species at the time (~6,000) [1,2,5].

In 2005, the IUCN and Conservation International (CI) held the International Amphibian Conservation Summit with two major achievements: the development of the Amphibian Conservation Action Plan (ACAP)

[1,5], published in 2007[6], and the formation of the IUCN's Amphibian Specialist Group (ASG). In 2007, after a meeting held in Panama by the IUCN SSC Conservation Breeding Specialist Group and the World Association of Zoos and Aquariums, the Amphibian Ark was formed, an initiative focusing in *ex-situ* amphibian conservation efforts [1,5]. This effort was followed by the formation of the Amphibian Survival Alliance (ASA) in 2011, a global initiative aimed to prevent amphibian extinctions, promote species and habitat conservation, foster international cooperation efforts and educate people about the importance of amphibians and their conservation (www.amphibians.org). The Action Working Groups (AWG) from ASA are working hard to reverse the effects of the amphibian crisis, create awareness on a global scale and promote research for the conservation of this entire class [1].

As a result of all these conservation efforts and research, we now know that amphibian populations around the world continue to experience drastic declines and even extinction. Out of the 7,391+ recognized amphibian species to date, 159 might have become extinct already, with 38 formally considered extinct, 1 extinct in the wild and 120 that have not been observed in the wild for several years now [7,8]. Forty two percent (3,100+) of the species are declining in numbers, especially in Latin America and the Caribbean, and another 26% (1,900+) species fall in one of the three International Union for the Conservation of Nature's (IUCN) (2014) categories of risk [9-13]. Compared to the percentages of threatened species for mammals (23%) and birds (12%), amphibians are facing an extremely rapid extinction crisis of a scale not previously seen in modern species [1,2].

Because of its richness and diversity of species, the order Anura (frogs and toads) shows the highest number of threatened species, compared to that of the Caudata (salamanders and newts) and Gymnophiona (caecilians) orders, with the Bufonidae, Leptodactylidae and Rhacophoridae families among the most threatened [14]. The genus *Atelopus* represents the largest in the Bufonidae family with 82 recognized species, an estimated 30 forms being considered for description as new species, 3 extinct species and 66 critically endangered, all distributed in 11 countries in Central and South America [2,10,12,15,16].

In 2005, La Marca et al., found that this genus had suffered the most drastic decline compared to all other amphibian genera, where 56% of all *Atelopus* species with enough data available have probably disappeared from their known habitats, given there are no records of observation since the year 2000. In 2008, Frost et al., reported that 84% of the *Atelopus* species were under critical risk. Chytridiomycosis (an infectious disease caused by a zoosporic fungus), climate change, pollution, habitat destruction and the pet trade are possible causes of these declines and extinctions [2,12,15-19].

The genus seems to be particularly vulnerable to chytridiomycosis infection. Thirty-two (32) *Atelopus* species have declined, and of these, 22 have disappeared from regions where the disease is present despite inhabiting protected areas [12,15]. In fact, the first documented cases of chytridiomycosis related to amphibian populations declines were found in *Atelopus* species during the early 1980's in Ecuador and in the early 1990's in the Cloud Forests of Monteverde, Costa Rica [15,18,20,21].

The Panamanian Golden Frog

The Panamanian Golden Frog (*Atelopus zeteki*, Anura: Bufonidae) -PGF- is an endemic species from Panama found in the provinces of Coclé and Panamá from low to mid elevations [12,22]. This is an iconic species in the Country and it is known for being deeply attached to the Panamanian culture [22,23]. The species is listed as Critically Endangered (CR) by the IUCN since 2004, the highest criteria for a species facing extinction; is under CITES Appendix I [24] and it is also protected under Panamanian law. PGF's are known for being one of the most charismatic and popular endangered amphibian species of the world [20]. This makes the Panamanian Golden Frog a good candidate to serve as a model species for the study and conservation of other *Atelopus* species.

The species was fairly common during the 1980's but recent reports suggest that remnant populations have disappeared from some, if not all of its known habitats [12]. Scientists even believe that, at the present, the species might be already extinct in the wild [25]. As with many other amphibian species of the world, PGF's are being affected by what it is considered the sixth great extinction in our planet. Great efforts are being implemented to save this and many other species from extinction. Captive populations in zoos and amphibian arks have become the only hope for PGF's to survive.

Conservation efforts

For a species facing such a risk, captive breeding programs seem to be the only road for the preservation of remaining populations [13,15,16,20,26]. As a matter of fact, the IUCN strongly encourages that "*all critically endangered and extinct in the wild taxa should be*

subject to ex situ management to ensure recovery of wild populations" [27]. Conservation initiatives such as Project Golden Frog (PGF), the El Valle Amphibian Conservation Center (EVAAC), the Amphibian Recovery and Conservation Coalition (ARCC) and the Amphibian Rescue and Conservation Project (ARCP) were created due to the general concern for the status of these species and have been established with the collaboration of national and international institutions for the conservation of endangered amphibians from the region, including PGF's [20,22]. Captive rescue programs like these have been proven successful for the preservation of highly endangered – almost extinct species such as the case of the Black Footed Ferret Recovery Plan that led to the production of more than 4000 ferrets in a period of 15 years and the start of a reintroduction program [28].

Reproductive physiology

There is very limited information available on the reproductive biology of PGF's or amphibian species in general. Panamanian Golden Frogs are believed to reach sexual maturity at two years old [23]. PGFs are external fertilizers that breed in or near streams from the end of November to January [29]. Females oviposit a long strand of eggs and attach them to the rocks in the streams where amplexed males fertilize the eggs in the water [29].

Maintaining viable captive breeding populations of amphibians is not trivial. There is still much to be learned about their ecology, physiology, genetics, and particularly, an urgent need for basic studies on their reproductive biology, key elements needed to manage these populations successfully. Captive populations have been created to confront the drastic declines amphibians are facing in the wild, however, these populations are facing now a new crisis: the captive breeding one [11,30].

The lack of knowledge is an obstacle when trying to stimulate natural reproductive behaviors or artificially reproduce amphibians in captivity [11,30]. Problems such as unsuccessful or asynchronous spawning, the absence of male breeding behavior, dystocia, low survival rates of eggs, embryos and juveniles, and diseases, add to the common problems captive populations face, such as inbreeding depression, genetic drift, low genetic diversity, and loss of evolutionary adaptability, among others. These issues make amphibian reproduction in captivity a difficult task [11,31,32].

Assisted Reproductive Technologies (ARTs)

Once the fundamental traits of the reproductive physiology have been characterized, it is possible to develop alternative solutions to enhance the reproduction of a given species in captivity. Assisted Reproductive Technologies (ART's) such as hormonal stimulation for the induction of spermiation and ovulation, cryopreservation and short term storage of sperm, oocytes and embryos (Genome Resource Banks), Artificial Fertilization (AF), and Intracytoplasmic Sperm Injection (ICSI), are convenient tools that help improve the management and success of captive amphibian populations [11,32,33].

Genome Resource Banks (GRB's) are frozen repositories of biological material where viable gametes; blood, tissues or DNA can be stored for decades [34-36]. GRB's have become a great tool for conservation biologists because they facilitate the exchange of genetic materials between captive populations, help preserve genes for longer generations, provide a resource to increase genetic viability, relieve space problems in breeding institutions by reducing the number of broodstock, eliminate quarantine issues, and become a continuous supply of biological materials [36].

In particular, sperm cryopreservation is an efficient approach to solve space problems by allowing institutions to keep fewer males in captivity and, at the same time, store its genetic diversity for generations. This approach has been successfully used for mammalian [36] and some amphibian species [32,37]. The development and implementation of Assisted Reproduction Technologies (ARTs) and Genome Resource Banking (GRB's) will help maintain healthy captive populations that will serve as an insurance policy for the species until the time comes for their reintroduction in the wild.

Experts have expressed an urgent need for ART's, including the implementation of cryopreservation and GRB formation, to ensure the long-term preservation of these amazing species. Meeting this priority requires urgently understanding the fundamental sperm physiology of PGF's and amphibians in general, including the sensitivity of these haploid cells to osmotic changes, exposure to cryoprotectants, freezing and thawing. There currently is no basic information available on the structure, physiology, collection or preservation of sperm from living golden frogs.

Sperm structure and functionality

For the successful application of sperm cryopreservation, it is necessary to first understand the structure and functionality of the sperm cells and how they behave when exposed to different extracellular environments. Parameters such as sperm viability, general morphology, acrosomal, mitochondrial and DNA integrity are essential traits to consider when studying sperm cells [38].

Distinct cellular structures react differently to intra and/or extracellular stressors [39]. Sperm response to osmotic and temperature changes depends on a variety of factors such as membrane composition, water and ion channels and signaling pathways, among

others [40]. Studies on the effect of osmotic stress on the biophysical changes of the sperm membrane indicate that alterations on the concentration of solutes in the extracellular environment lead to structural damage, increased water influx and lysis [41].

Most of the information available comes from studies in mammalian spermatozoa in humans [42], mice [43] and boars [44], among others. At the moment, there are no published records on these parameters for *Atelopus* sperm cells and just a few for a handful of amphibian species [45-48].

Sperm collection and cryopreservation

There are no published studies on the collection or cryopreservation of *Atelopus* spermic urine. Cryopreservation of sperm has been attempted for several amphibian species such as *Xenopus laevis, X. tropicalis, Rana sylvatica, R. temporaria, R. pipiens, Bufo americanus, B. marinus, B. fowleri, B. baxteri, B. boreas, Litoria fallax, L. lesueurii, L. phyllochroa, L. peroni, L. subglandulosa, L. verreauxii, Eleutherodactylus coqui* and *Myobatrachia* spp. [11,49-61]. However, studies on the morphology, structure and physiology of sperm cells have only been reported for just a handful of these species. Most of the studies attempting amphibian sperm cryopreservation have not previously characterized the sperm traits or even evaluated the physiological changes sperm cells undergo when exposed to these conditions.

Anurans with external fertilization, like PGF's, excrete the sperm in the urine, which makes its collection easier compared to higher vertebrates [11]. Sperm samples from live animals can be collected through the use of intracoelomic injections of stimulating hormones such as GnRH and hCG and a catheter, which have been proven effective for some amphibian species [11,32,48,52,53,55,61-67]. Another technique for sperm collection from live

animals after hormonal stimulation, especially for toads, is the stimulation of the urinating defense mechanism triggered by threatening events [11].

Most of these studies have accomplished cryopreservation of testicular sperm, extracting the cells from the testicles of euthanized males [11,33,50,51,55,57,58,67,68]. This is a questionable methodology when colleting sperm from endangered species such as PGF's, where every animal is critical for the establishment and success of captive populations [11].

Cryopreservation of spermatozoa collected from live anurans using different combinations of penetrating and non-penetrating cryoprotectants has been proven difficult [53]. Researchers believe that the activation and inactivation of sperm when exposed to osmotic fluctuations, and the impact of centrifugation, might be causing structural damages in the cells, thus, resulting in low recovery of motile or even viable sperm [11,55,61,68,69].

Osmolality and sperm activation

In anurans and fish, motility and osmolality are closely associated since sperm responds to osmotic changes in the extracellular environment [70-73]. In other species, osmolality and/or changes in ion concentrations are also thought to trigger motility [71,73-75]. In anurans, as in freshwater fish, the cells are inactive (immotile) inside the testis (isotonic environment) that have an osmolality of ±300 mOsm/kg [32,55,69,71,75-78]. When exposed to hypotonic environments such as urine or water, sperm becomes active (motile); inactivation occurs when the cells are exposed to hypertonic environments [11,32,37,50,61,75,76,79,80].

Osmotic changes are known to have a detrimental effect on anuran and fish sperm motility and viability [69]. Cryopreservation of spermic urine involves exposing the cells to

even more drastic osmotic changes in three activation-inactivation steps [11]: inactive testicular sperm activates when released in the urine; active sperm in the urine then inactivates one more time when exposed to the high osmolality of cryoprotectants; inactive cryopreserved sperm activates again when thawed and diluted. This susceptibility to osmotic stress may be one of the causes of poor post-thaw sperm recovery; yet, there is little information available on the impact these changes have in the structure and functionality of amphibian sperm cells and their application on the development of cryopreservation protocols.

In Bufonids, sperm motility has been associated with the presence of a distinctive structure in the cell: the Mitochondrial Vesicle (MV). This is a cytoplasmic droplet like structure that holds a high concentration of mitochondria, and it is located in the mid to lower part of the sperm head [28,47,48,55,61,68,81]. The MV (Chapter 2, Figure 5) seems to be a very fragile structure. Exposure to osmotic and temperature changes, as well as to mechanical stress (maceration or centrifugation), might cause the loss or damage of the MV [61]. The presence of such structure makes sperm manipulation in Bufonids even more challenging. Preliminary observations in our laboratory indicate the presence of such a structure in PGF's spermatozoa.

Besides the structural damages caused by osmotic fluctuations or loss of the MV, these changes can also trigger DNA damage that have been associated to low post-thawed sperm viability when attempting cryopreservation [82,83]. Generation of reactive oxygen species (ROS), intracellular ice formation, osmotic imbalances, and cooling rates have been shown to induce damages in membranes, organelles and sperm DNA in trout, bream and different mammal species such as human, mouse, horse and pig [82-87]. DNA

fragmentation has been associated to increases in intracellular calcium levels, production of ROS and interactions with genotoxic compounds [83]. Understanding the effect osmotic changes have in the integrity of sperm DNA is critical for the development of accurate handling procedures and cryopreservation protocols.

Sperm motility activation pathways

Extracellular stressors, such as osmotic changes, pH and the concentration of ions, have the ability to disturb the structure and composition of cellular membranes and therefore, affect the signaling pathways responsible for numerous vital processes [40]. Osmotic shock and the concentration of Ca^{2+} and K^+ have been associated with sperm motility activation in some species of fish such as cyprinids, tilapia and puffer fish, and in amphibians such as *Xenopus laevis*, *Rana sylvatica* and *Rana pipiens* [69,76,77]. Changes in the concentration of K^+ have been related to changes in the polarization of the membrane in trout, possibly triggering the signaling pathway responsible for sperm activation [88].

Activation of sperm motility has been associated with a signaling pathway involving increasing concentrations of intracellular cyclic adenosine monophosphate (cAMP) and Ca^{2+}, and the subsequent phosphorylation of specific proteins by cAMP-dependent protein kinases in some species of mammals, including humans; in salmonids, rainbow trout and sea urchins, osmotic changes have been known to trigger the activation of this same pathway [77,89,90]. Nevertheless, for some species of fish such as sea bass and striped bass, and in bulls, the activation of sperm motility is independent of the cAMP and Ca^{2+} pathway [91]. The sperm motility activation pathway for *Atelopus* species is unclear. Understanding the process governing the activation of sperm in this species

would aid in the development of suitable cryoprotectants, enhancing the probabilities of obtaining higher concentrations of viable post- thawed sperm cells.

All the information provided above leads us to conclude that there is still much to learn and understand about the reproductive biology of *A. zeteki* for its urgent application in captive breeding programs. The fact that PGFs have external fertilization and its sperm cells activate upon contact with hypo-osmotic aquatic environments, lead us to hypothesize that the motility activation as well as the viability of *Atelopus zeteki*'s spermatozoa are regulated by osmotic changes of the extracellular liquid environment. This study will assess the structural and functional traits of *Atelopus zeteki* spermatozoa, determine the effect of hormonal stimulation on the functionality of the cells, evaluate the effect of osmotic changes on the viability of *A. zeteki* sperm for the future implementation of accurate protocols the species sperm cryopreservation. The use of innovative techniques such as collection of sperm from live specimens and TUNEL Assay will help us evaluate several parameters required to understand *A. zeteki* sperm structure and functionality. Taking into consideration that there is no available information on *Atelopus zeteki* sperm or reproductive physiology, knowledge gained from our observations can be applied for the conservation and captive reproduction of other endangered *Atelopus* species.

Chapter 2: <u>Effect of hormonal stimulation on the production of spermatozoa in the Panamanian Golden Frog (*Atelopus zeteki*).</u>

Abstract

Assisted Reproductive Technologies are necessary for the successful implementation of captive breeding programs. Our aim was to characterize the general parameters of *Atelopus zeteki* spermatozoa and to assess the effects of hormonal stimulation on sperm concentration, motility parameters, cell morphology and DNA integrity, as well as to determine the effect of seasonality on the hormonal stimulation response. Sperm samples were obtained after IP injection of GnRH (0.1, 0.2 and 0.4 μg/μl), hCG (0.5 and 1.0 IU/ μl) and Amphiplex (0.4 μg of GnRH-A and 10 μg of Metoclopramide hydrochloride) and sperm parameter were determined. Our results revealed *A. zeteki* successfully produces spermatozoa after stimulation with all hormones and concentrations, with Amphiplex stimulating the production of higher sperm concentrations ($p<0.05$). All hormone treatments produced sperm with >60% motility and FPM, not altering the osmolality of the spermic urine. Nevertheless, hormonal stimulation did significantly increase the pH of spermic urine ($p<0.05$) compared to controls. The general morphology of the cells was described, with *A. zeteki* spermatozoa presenting a similar morphology to that of other anuran species. Hormone treatments had no effect on the morphology and DNA integrity of the cells. Seasonality did not affect the hormonal stimulation response in the sperm concentration of *A. zeteki* spermatozoa.

Introduction

Amphibian populations around the world are experiencing drastic declines and even extinction [11,13,64,65,92,93]. The Panamanian Golden Frog (PGF), (*Atelopus zeteki*, Anura: Bufonidae), an endemic species from Panama that has not been observed in the wild for at least five years [9,22,94], is listed as Critically Endangered (CR) by the IUCN since 2004, the highest criteria for a species facing extinction; and it is also protected under Panamanian law [12,95].

For a species facing such a threat, captive breeding programs appear to be the only path for the preservation of remaining populations [13,15,16,20,26,66]. The lack of knowledge on reproductive physiology for many species turns into a great obstacle when trying to stimulate natural reproductive behaviors or artificially reproduce amphibians in captivity [11,30,66,96]. Complications such as unsuccessful or asynchronous spawning, the absence of male breeding behavior, dystocia, low survival rates of eggs, embryos and juveniles, and diseases, added to the common problems captive populations face, such as inbreeding depression, genetic drift, low genetic diversity, and the loss of evolutionary adaptability, among others, make amphibian reproduction in captivity a difficult task [11,32,97].

Assisted Reproductive Technologies (ART's) such as hormonal stimulation for the induction of spermiation and ovulation, cryopreservation and short term storage of sperm, oocytes and embryos (Genome Resource Banks), Artificial Fertilization (AF), and Intracytoplasmic Sperm Injection (ICSI), are convenient tools that aid to improve the management and success of captive amphibian populations [11,32,96,98].

Among the ART's toolkit, hormonal stimulation is an efficient technique that allows the manipulation of the reproductive cycle when adequately standardized for a species.

The technique is achieved by the administration of exogenous hormones to males and/or females for the collection of gametes (spermatozoa and oocytes) that can later be utilized for the application of other methods such AF, ICSI or cryopreservation [11,32,63,64,98].

The reproductive biology of anurans is under the control of the Hypothalamic-Pituitary-Gonadal Axis (HPG) which presents a consistent basic structure among most tetrapods [64,99] and it is regulated by the production of Gonadotropin-Releasing Hormone (GnRH) [66,100-103]. GnRH is a hypothalamic neuropeptide that acts on the anterior lobe of the pituitary gland, stimulating the biosynthesis and secretion of the sex hormones: Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), both glycoproteins that share one common α and two different β chains [30,32,63,64,67,98,99,103-115]. FSH and LH in turn regulate and promote the production of sex steroids and gametogenesis [32,63,98,99,103,116]. The role of FSH in amphibian reproduction is poorly understood; whereas is better known that LH, by acting on the Leydig cells in the testis, triggers spermiation [64]. Both, oviposition and spermiation, typically follow an increase in the production of LH [30].

There are several studies on diverse species of amphibians exploring the use of exogenous hormones for the collection of spermatozoa from live animals, where hormones, doses, concentrations and results vary distinctively among species [11,30,32,37,48,52,53,61,63,66,67]. The most utilized include GnRH-A [des-Gly^{10}, D-Ala^6, Pro-NHEt^9], Human Chorionic Gonadotropin (hCG) and hormone cocktails including GnRH and a dopamine antagonist such as metoclopramide, domperidone or pimozide [30,61,63,66,67,74,108,117-122].

Responses to hormone treatments differ among species [98]. In 1998, Waggener and Carroll treated eight species of anurans with GnRH, successfully obtaining spermatozoa

from *Ceratophrys ornara*, *Ceratophrys cranwelli*, *Pysicephalus adsperus*, *Lithobates pipiens*, *Xenopus laevis*, *Lepidobatrachus llanensis* and *Lepidobatrachus laevis* but not *Ceratophrys cornuta*. Mann et al., 2010, reported successful spermiation in *Litoria raniformes* after stimulation with GnRH. Sperm samples have also been collected utilizing this same hormone from *Bufo americanus*, *Bufo baxteri* [81,123], *Bufo valliceps* [124], *Pseudophryne guentheri* [67] and *Pseudophryne corroboree* [63].

Human Chorionic Gonadotropin (hCG), a gonadotropin produced in the human placenta, circumvents the HPG axis, operating directly on the gonads [63,66,98]. The effect of hCG on the induction of spawning results from this hormone binding to the same receptors as LH [125,126]. Sperm samples have been successfully obtained with the use of hCG from *Xenopus laevis*, *Xenopus tropicalis*, *Bufo marinus*, *Bufo arenarum*, *Litoria chloris*, *Litoria aurea*, *Lithobates pipiens*, [32,52,66,81,108,121,127], *Bufo fowleri* [62] and *Pseudophryne corroboree* [63]. Examples of the diversity in the responses of anurans to hormone stimulation are the cases of *P. corroboree* and *B. fowleri. Pseudophryne corroboree* responds positively to the treatment with both hormones, however, GnRH stimulates the production of significantly higher numbers of sperm than hCG [63]. On the other hand, *B. fowleri* responds better to the treatment with hCG than GnRH [62].

Studies in fish, amphibians, birds and mammals suggest the existence of an inhibitory mechanism disturbing the biosynthesis of gonadotropins [30,32,64,114,118,128,129]. The central dopaminergic system, through the DA-2 (dopamine) receptors, is associated with fluctuations on the GnRH and the GnRH-R (receptor) gene expression, and consequently, in LH secretion [64,66,74,115,117]. Studies in fish suggest that blocking of the DA-2 receptors increases LH release [30,64,66,118,129,130]. Moreover, research carried out by Sotowska-

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Brochocka et al., in 1994 concluded that treatment with a DA-2 agonist inhibits the production of LH in *Rana temporaria*. More recent studies by Browne et al., in 2006 and Trudeau et al., in 2010 reported that the use of dopamine antagonists like Pimozide and Metoclopramide in combination with progesterone and GnRH or GnRH alone increased spawning in *Anaxyrus fowleri* and *Lithobathes pipiens*, respectively. Besides sperm concentration, other parameters such as motility, cell morphology, osmolality and pH are essential traits to be assessed for the evaluation of sperm quality [70].

Sperm motility is usually considered an indicator of fertilization success, frequently associated with the type of fertilization and the spawning environment of a given species, and believed to be species specific [72,76,78,131]. In anurans and fish, motility and osmolality are closely associated since sperm responds to osmotic changes in the extracellular environment [70-73]. In other species, osmolality and/or changes in ion concentration are also thought to trigger motility [71,73-75]. In anurans, as in freshwater fish, the cells are inactive (immotile) inside the testis (isotonic environment) that have an osmolality of ±300 mOsm/kg [32,55,69,71,75-78]. When exposed to hypotonic environments such as urine or water, sperm becomes active (motile); inactivation occurs when the cells are exposed to hypertonic environments [11,32,37,50,61,75,76,79,80].

Osmotic changes are known to have a detrimental effect on anuran and fish sperm morphology and viability [69,76]. Exposure to hypotonic environments causes osmotic shock resulting in the swelling and structural disruption of the cells, and therefore causing morphological abnormalities [73,75]. A study by Perchech Poupard et al., in 1997 in carp spermatozoa revealed the effect of osmotic stress in the head volume of these cells; taking as a normal volume that in isotonic environments (300 mOsm/kg inside the testis),

a progressive swelling was observed when the cells were exposed to lower osmolalities, as well as a continuous shrinkage of the heads when the osmolality of the extracellular environment was increased, returning to normal volumes upon reaching 300 mOsm/kg. The study also revealed that the nuclei of permeabilized cells exposed to low osmolalities ruptures while tails appeared to remain intact. This demonstrates the strong effect the osmolality of the extracellular environment has on the sperm cell's morphology and integrity [69,74,75].

Ejaculate pH is another important characteristic to consider when assessing sperm traits since changes in normal values can affect the functionality of the cells [74]. Moreover, there is some evidence indicating that extracellular pH alone or in combination with osmolality and the extracellular concentration of ions such as K^+ might affect sperm intracellular pH and motility in fish, amphibians, boar and ram [73,75,76,78]. A study by Takai and Morisawa in 1995 in marine and freshwater fish revealed that intracellular pH (pH_i) corresponds to extracellular pH (pH_o) even if external $[K^+]$ varied. The authors also indicate that low pH_i arrested the initiation of motility with very high $[K^+]$ required to activate it, whereas higher pH_i initiated sperm motility even in the presence of very low $[K^+]$. Another study by Christensen et al., in 2004 assessed the effect of pH and dilution on the motility of *Xenopus laevis* spermatozoa, indicating a clear influence of pH on sperm motility. Results from this study reported a cessation of motility when *X. laevis* spermatozoa were exposed to acidic environments and an increase in motility with exposure to more alkaline environments. Other studies have reported similar effects of pH on sperm motility in different species such as sea urchin, trout and newts [65,76,132].

DNA integrity is another important trait to consider when evaluating sperm quality, since intact DNA is essential for fertilization and embryo development [133,134]. Inside the nucleus of mature spermatozoa, chromatin is highly stable and tightly packaged in association with Sperm Nuclear Binding Proteins (SNBP) such as protamines, protamine-like proteins and histone-like proteins, proteins that replace histones during the late stages of spermatogenesis [135-137]. In amphibians, the most common type of SNBP are protamine-like and histone-like proteins [135]. This packaging is thought to protect the DNA from oxidative damage or stress during the process of fertilization [133,137]. Sperm DNA fragmentation has been associated with oxidative stress, apoptosis or deficiencies in DNA condensation and affects other parameters such as motility, morphology and fertilization [133,136,138].

A study by Huang et al in 2005 revealed that DNA fragmentation in men is associated with abnormal sperm morphology and decreased motility, suggesting the importance of evaluating DNA fragmentation along with all other routine sperm quality parameters for the application of ART's. Another study by Dietrich et al in 2005, analyzed the effect of induced DNA damage by UV irradiation in rainbow trout spermatozoa, revealing a negative effect in motility and embryo development. Thus, DNA integrity is essential for sperm functionality.

All of the traits mentioned above affect sperm structure and therefore, functionality. Sperm structure or morphology is also an important indicator of male fertility [139,140]. Sperm morphology of some anurans is thought to be associated with their spawning environment and shows coevolution with egg morphology [131]. In the case of anurans with external fertilization, after a short period of time when spawned eggs come in contact

with water, hydration of the jelly layers surrounding the egg makes it impenetrable for sperm cells [11], thus normal morphology that facilitates the rapid swimming of spermatozoa to reach the egg in time before egg swelling and before loss of sperm viability due to osmotic shock is essential for fertilization.

There is very limited information on the reproductive ecology of PGF's or amphibian species in general. Panamanian Golden Frogs are believed to reach sexual maturity at two years old. PGFs are external fertilizers that breed in or near streams, they are believed to be seasonal breeders since amplexant pairs and eggs have been observed from late November to January [29]. Females oviposit a long strand of eggs and attach it to the rocks in the streams where amplexed males fertilize the eggs in the water [29].

Our hypothesis was that the use of exogenous hormonal stimulation impacts the quantity and quality of excreted spermatozoa in the highly endangered Panamanian Golden Frog. Our specific aims were to characterize the effect of the hormonal stimulation treatments specifically on: 1) sperm concentration, motility and Forward Progressive Motility (FPM); 2) osmolality and pH; 3) morphology and DNA integrity; and the effect of seasonality on the hormonal response. The main purpose of the study was to compare the effect of hormonal treatments on *A. zeteki* sperm functionality and quality, and therefore determine the most efficient hormone treatment, in terms of sperm concentration and integrity that stimulates the production and facilitates the collection of PGF spermatozoa.

Materials and Methods

Animals and Approvals

Thirty adult males *Atelopus zeteki* (>2 year old, average weight of 3.81±0.41 g) were obtained from the Species Survival Plan (SSP) population from the Maryland Zoo in Baltimore, MD. Animal Use Protocols were submitted for the Institutional Animal Care and Use Committee (IACUC) approval from the University of Maryland, the Smithsonian National Zoological Park (SNZP) in Washington, D. C., and Maryland Zoo in Baltimore, MD. After obtaining IACUC approvals, the animals were transferred from the Maryland Zoo to the SNZP and were placed 30 days under quarantine. One individual died during quarantine and three more died over the course of the next two years.

Six terraria (Exo-Terra Terrarium, 24"x18"x24") were adapted and designed to accommodate 5 males per tank. Each tank was fitted with live potted plants and live moss as substrate. The recommended humidity in the tanks (60% to 90%) was achieved with the use of an automated misting system that works with reverse osmosis filtered water and that was set up to mist once every hour for two minutes. Excess water was discarded utilizing a flow through drainage system. Temperature ranged from 20 to 22 °C with the use of a window unit air conditioner. All frog terraria were supplied with a UVb Light System, (Zoomed's 10.0 T5 High Output Bulbs) to avoid hypovitaminosis D_3 and help with proper bone growth [141].

Frogs were fed four times per week. Food items included; one-week-old crickets dusted with a calcium carbonate powder containing vitamin D_3, wingless fruit flies and bean beetles. Items were fed on a rotating basis.

For identification purposes, four photographs of each animal were taken to distinguish males utilizing their spot patterns and an alpha-numeric code was assigned to each as follows: PGF01 to PGF30, where PGF stands for Panamanian Golden Frog.

Hormone Stimulation and Sperm Collection

Exogenous hormonal induction was used to stimulate the production of spermic urine in males *Atelopus zeteki*. Production of hormonally induced spermic urine differs from one species to the other [63,65,67,119]. Testing different hormones and concentrations helped determine what treatment worked better for the collection of highly concentrated spermic urine for this species in particular.

Hormonal stimulations were administered via intracoelomic injection with the help of a Veterinarian from the National Zoo [11,62]. Treatments were carried out once a week for twelve weeks by stimulating groups of three randomly selected males with 40 µl of one of six hormone treatments and one randomly selected male as control, making a total of 4 individuals per week and of eight individuals by treatment. Males used as controls were stimulated with an injection of 40 µl of Amphibian Ringer Solution (ARS) or no injection at all. Each group received one of the following treatments twice: **1)** Treatment 1: 1 µg of GnRH-A [des-Gly[10], D-Ala[6], Pro-NHEt[9]] (Sigma-Aldrich Corporation, St. Louis, MO, USA) per gram of bodyweight, 2**)** Treatment 2: 2 µg of GnRH-A [des-Gly[10], D-Ala[6], Pro-NHEt[9]] per gram of bodyweight, **3)** Treatment 3: 4 µg of GnRH-A [des-Gly[10], D-Ala[6], Pro-NHEt[9]] per gram of bodyweight, **4)** Treatment 4: 5 IU of hCG [CG5 Chorionic

gonadotropin human] (Sigma-Aldrich Corporation, St. Louis, MO, USA) per gram of bodyweight, **5)** Treatment 5: 10 IU of hCG [CG5 Chorionic gonadotropin human] per gram of bodyweight, and **6)** Treatment 6: a cocktail of 0.4 μg of GnRH-A [des-Gly^{10}, D-Ala^6, Pro-NHEt^9] (Bachem Americas Inc., Torrance, CA, USA) and 10 μg of Metoclopramide hydrochloride (Sigma-Aldrich Corporation, St. Louis, MO, USA) per gram bodyweight (Amphiplex Method) (Trudeau et al., 2010). Dose selection was based on approximates for those that have successfully promoted spawning in other species of amphibians [30,81,119]. After injections, males were placed inside ventilated plastic containers with water soaked paper towel on the bottom and covered from light to reduce stress. Spermic urine was collected by gently inserting a small catheter (0.023" I.D. x 0.039" O.D., Micro Medical Tubing, 85 Durometer Vinyl, Scientific Commodities Inc., Lake Havasu City, AZ, USA) in the cloacae [65]. Sperm collections were carried out during seven different time points as follows: 0.5h, 1.5h, 2.5h, 3.5h, 4.5h, 5.5h and 24h post injection.

Sperm processing and analysis

Basic metrics were utilized to define the general characteristics of *A. zeteki's* spermic urine. Total volume of excretion was determined with the use of a micropipette. Sperm concentration was assessed with the use of a hemocytometer; 10 μl of spermic urine were added to the chamber and counted following the 5 square rule [142]. The osmolality and pH of the spermic urine were obtained with the use of an osmometer (Vapro Osmometer 5520, Wescor, Inc, South Logan, Utah, USA) and pH strips (pH-indicator strips pH 5.0 - 10.0, EMD Millipore, Billerica, MA, USA) respectively. Percentage of sperm motility was determined by counting all the cells with flagellar movement from a total of 100

cells analyzed under an Olympus BX41 microscope at a 400X magnification. Forward progresive motility (percentage) was obtained by counting all the cells expressing forward motility relative to the 100 cells that were expressing any kind of flagellar movement.

Morphology

Morphology was assessed by fixing 10 μl of the samples in 80 μl of 4% paraformaldehyde solution in PBS. Fixed cell were stained with Coomassie Blue (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) and mounted with Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, PA, USA). Sperm morphology was evaluated at a 1000X magnification under an Olympus BX41 microscope in two different ways: 1) normal and abnormal general classification and 2) specific assessment by morphological trait. For the first assessment, a maximum of 100 cells were assessed for every sample and classified as normal if every morphological parameter of the cell was intact (acrosome, head and tail) and as abnormal if at least one of the parameters was not intact. For the second evaluation, a maximum of 100 cells per sample were evaluated and assessed for acrosomal integrity [normal (intact) and abnormal (not intact, missing)], head [normal (intact) and abnormal (bent, broken)] and tail [normal (intact) and abnormal (coiled, with cytoplasmic droplet or broken)]. Images of the cells were acquired with a SPOT RT3™ Color Slider microscope camera and the SPOT Advanced Software (SPOT Imaging Solutions, Sterling Heights, MI, USA). Measurements of the head and tail were obtained from 100 cells from different individuals with the SPOT Advanced Software segmented line measuring tool.

Testicular spermatozoa from eleven euthanized males from another study were also

used as a control for the morphology assessment. Testes were removed after dissection, macerated in 1 mL of Amphibian Ringer Solution and fixed with 4% paraformaldehyde. Cells were analyzed as described above.

Identification of the nucleus was accomplished using Hoechst 33342 and Propidium Iodide (PI) staining (Sigma-Aldrich Corporation, St. Louis, MO, USA). An aliquot of 10 µl of cells previously fixed in 4% parafolmaldehyde was stained with 3 µl of either Hoechst (excitation/emission of 338/505 nm, 50 mg/ml) or PI (absorption 493 nm, 50 mg/ml) and analyzed under a fluorescence Olympus BX41 microscope (400X). For the mitochondrial vesicle staining, 1 µl of 100 nM MitoTracker® Green FM (Life Technologies, Grand Island, NY, USA), excitation/emission of 490⁄516 nm, was added to 10 µl of fresh spermatozoa. After 15 minutes, cells were fixed in 4% parafolmaldehyde and analyzed under fluorescence Olympus BX41 microscope (400X).

DNA Integrity

Sperm DNA fragmentation was determined with the use of the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique (DeadEnd™ Colorimetric TUNEL System, Promega Corporation, Madison, WI, USA), with an in situ cell detection kit, following the manufacturer's instructions. Ejaculated spermatozoa used in this assay were previously fixed with 4% paraformaldehyde and kept at 4°C until analyzed. Slides were prepared by smearing the samples and letting them air-dry. Slides were then gently rinsed twice with PBS for 5 minutes. Cells were permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature. Slides were again gently rinsed twice with PBS for 5 minutes followed by incubation with the equilibration buffer for 10 minutes at room temperature. Then, 100 µl of previously prepared rTdT reaction mix

were added to the slides. After covering with plastic coverslips, slides were incubated at 37°C for 60 minutes in a humidified chamber. The reaction was terminated by removing the coverslips and adding 200 μl of 2X SCC to each slide using a pipette, incubating them for 15 minutes at room temperature. Slides were rinsed twice with PBS for 5 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 5 minutes. Slides were again rinsed twice with PBS for 5 minutes followed by incubation with 200 μl of Streptavidin Horseradish Peroxidase for 30 minutes. Slides were rinsed twice with PBS for 5 minutes and developed with 3,3′-diaminobenzidine. Slides were mounted with Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, PA, USA) and analyzed under an Olympus BX41 microscope. One hundred cells were counted per slide and classified as intact or fragmented, those with DNA fragmentation were stained dark brown.

Testicular spermatozoa from 11 euthanized males from another study were used as a control for the TUNEL assay. Testes were removed after dissection, macerated in 1 mL of Amphibian Ringer Solution and fixed with 4% paraformaldehyde. Cells were analyzed following the same procedure described above.

Seasonality

To assess if seasonality affected *A. zeteki*'s sperm concentration, spermic urine osmolality and pH after hormonal stimulation, we divided the year in four season of three months each, starting with season 1 from January to March, season 2 from April to June, season 3 from July to September and season 4 from October to December. We compared average sperm concentrations, spermic urine osmolality and pH during the production peak of all hormone treatments among seasons during a period of two years.

Statistical analysis

For each hormonal treatment, sperm concentration peaks (time points with the most elevated sperm concentrations) were determined using a cutoff value that was calculated averaging the total sperm concentration of all time points for each treatment plus one time the standard deviation (SD). The time points with sperm concentrations equal or above the cutoff were removed (as part of the peak) and a new cutoff value was calculated with the remaining time points. This process was repeated until there was no time point left with a sperm concentration equal or higher than the cutoff. All values equal or higher than the cutoffs were considered the most elevated sperm concentrations and part of the peak for each hormone treatment [143] (Table 1).

Table 1. Calculation of sperm concentration peaks by hormonal trial. The symbol (/) indicates no more time points were selected for a specific cutoff.

HORMONE	n	Overall Mean cells/ml	Cutoff 1 cells/ml	Peak Time Point Selected	Cutoff 2 cells/ml	Peak Time Point Selected	Cutoff 3 cells/ml	Peak Time Point Selected	Cutoff 4 cells/ml	Peak Time Point Selected	Total # of Time Points/ Peak
GnRH –A 0.1 µg/µl	6	$9.15 \times 10^5 \pm 7.60 \times 10^5$	1.68×10^6	1	1.38×10^6	2	5.04×10^5	0	/	/	3
GnRH-A 0.2 µg/µl	6	$1.02 \times 10^6 \pm 6.61 \times 10^5$	1.68×10^6	2	1.26×10^6	1	9.93×10^5	1	6.75×10^5	0	4
GnRH-A 0.4 µg/µl	6	$1.94 \times 10^6 \pm 7.51 \times 10^5$	2.69×10^6	1	2.45×10^6	1	2.22×10^6	0	/	/	2
hCG 0.5 IU/µl	6	$1.0 \times 10^6 \pm 6.47 \times 10^5$	1.65×10^6	1	1.46×10^6	1	1.31×10^6	1	1.10×10^6	0	3
hCG 1 IU/µl	6	$1.06 \times 10^6 \pm 6.84 \times 10^5$	1.74×10^6	1	1.01×10^6	3	6.73×10^5	0	/	/	4
Amphiplex	6	$2.23 \times 10^6 \pm 1.81 \times 10^6$	4.04×10^6	2	2.54×10^6	0	/	0	/	/	2

All statistics were carried out utilizing the GraphPad Prism 6.05 software (GraphPad Prism 6.05, 2014, GraphPad Software, Inc., La Jolla, CA, USA).

Sperm concentration peaks were then used to calculate the effect of hormone treatments on sperm concentration using One-Way ANOVA followed by a Tukey's test for multiple comparisons to determine differences among the treatments.

Osmolality, pH, motility and FPM were also compared among hormone treatments using One-Way ANOVA followed by a Tukey's test for multiple comparisons to determine differences among the treatments.

The effect of hormones on the morphology of the cells was evaluated using The Kruskal-Wallis test and One-Way ANOVA followed by Dunn's and Tukey's test for multiple comparisons to determine differences among the treatments.

The seasonality effect on the production of sperm was assessed using One-Way ANOVA followed by a Tukey's test for multiple comparisons to determine differences among the seasons.

The Brown-Forsythe test was conducted to determine homogeneity of variances for all the One-Way ANOVA analysis. When variances showed significant heterogeneity, non-parametric tests were utilized.

A Spearman correlation test was computed to assess the relationship between osmolality and pH, FPM, and between pH and osmolality, Motility and FPM.

DNA fragmentation was compared among hormone treatments using One-Way ANOVA followed by a Tukey's test for multiple comparisons to determine differences among the treatments.

All statistical analysis were considered significantly different at $p<0.05$.

Results

Hormone Stimulation and Sperm Collection

Panamanian Golden Frog spermatozoa was successfully collected from all the hormone treatments during a period of 24 hours, starting at the first time point 30 minutes post-injection, followed by 5 hourly collections and a final one at hour 24[t] post-injection.

Sperm concentration ranged from 10^4 to 10^6 cells/ml. Spermic urine volume fluctuated from 10 to 270 µl, osmolality from 56 to 130 mOsm/kg and pH from 6.8 to 8.5. The average temperature of collection was 21.15±0.05 °C.

Table 2 shows the post-injection time points with highest sperm concentrations per hormone treatment following the procedure outlined in Table 1. The peak of sperm production for each treatment includes: 1) GnRH-A 0.1 µg/µl: 2h peak; 2) GnRH-A 0.2 µg/µl: 3h peak; 3) GnRH-A 0.4 µg/µl: 1h peak; 4) hCG 0.5 IU/µl: 2h peak; 5) hCG 1 IU/µl: 3h peak; and 6) Amphiplex: 1h peak.

Table 2. Sperm concentration peak by hormone treatment. Stars indicate the time points corresponding to the sperm concentration peak for each of the hormone treatments.

SPERM CONCENTRATION PEAK BY HORMONE							
Time Point (hours post-injection)							
	0.5	1.5	2.5	3.5	4.5	5.5	24
HORMONE							
GnRH-A 0.1 µg/µl		★	★	★			
GnRH-A 0.2 µg/µl		★		★	★	★	
GnRH-A 0.4 µg/µl			★	★			
hCG 0.5 IU/µl			★	★	★		
hCG 1 IU/µl			★	★	★	★	
Amphiplex				★	★		

Sperm processing and analysis

There was a significant difference in sperm concentration among hormone treatments ($F_{(7,20)}$ = 151.4, $p< 0.0001$). Out of the six treatments, GnRH-A 0.4 µg/µl ($2.65\text{x}10^6 \pm 2.12\text{x}10^5$, $p<0.0001$) and Amphiplex ($4.45\text{x}10^6 \pm 7.1\text{x}10^4$, $p<0.0001$) produced significantly higher sperm concentrations during the peaks of sperm production, with

Amphiplex producing 1.6 times more sperm than GnRH-A 0.4 µg/µl. There were no differences among the rest of the treatments or between the controls (p >0.05). The two controls did not promote spermiation in any of the individuals, indicating that neither an ARS injection nor placing the males in water will stimulate the production of spermatozoa in the Panamanian Golden Frog (Fig 1).
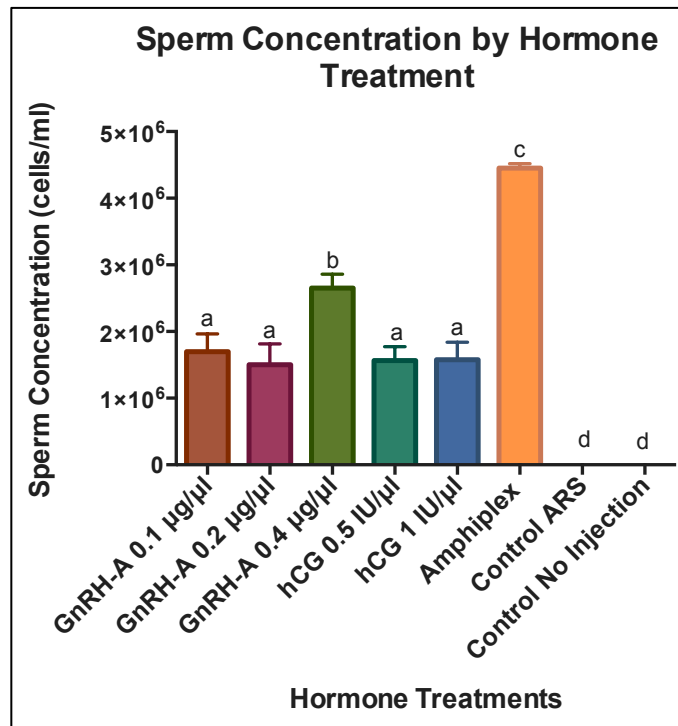
Figure 1. Comparison of sperm concentration during peaks by hormonal treatment. Mean ± SD sperm concentration (cells/ml) of six hormone treatments and two controls, n=24, 6 replicates. ANOVA ($F_{(7,20)}$ = 151.4, $p < 0.0001$), Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

Percentage of sperm motility varied among hormone treatments ($F_{(5,12)}$ = 4.308, p= 0.0178), specifically between GnRH-A 0.4 µg/µl and hCG 1 IU/µl (Mean ± SD 87.25

± 0.77%, 97.80 ± 2.44%; adjusted p = 0.0446) with the latter resulting in a higher percentage of sperm motility. There were no differences among the remaining treatments ($p > 0.05$) (Fig 2).
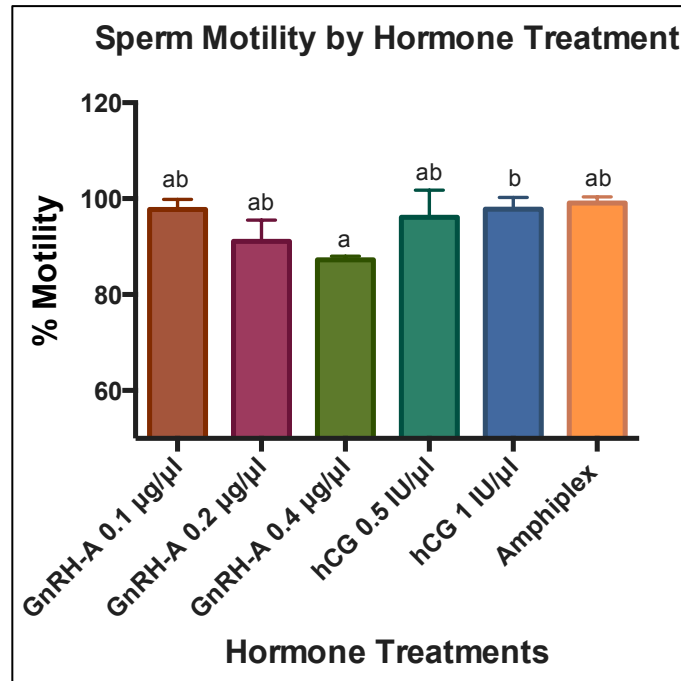
Figure 2. Comparison of percentage sperm motility among hormone treatments. Mean ± SD percentage sperm motility of six hormone treatments, n=27, 6 replicates. ANOVA ($F_{(5,12)}$ = 4.308, p = 0.0178), Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

Percentage sperm forward progressive motility (FPM) also showed differences among hormone treatments ($F_{(5,12)}$ = 4.337, p = 0.0174). FPM diverged between GnRH-A 0.2 μg/μl and hCG 1 IU/μl (Mean ± SD 68.90 ± 7.31%, 95.38 ± 2.10%; adjusted p = 0.0169) with hCG 1 IU/μl showing higher FPM. There were no differences among the additional treatments ($p > 0.05$) (Fig 3).
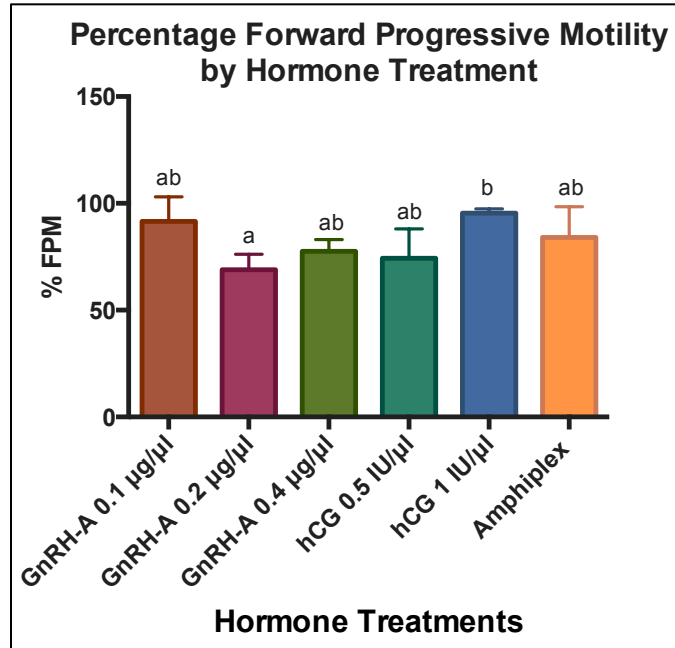
Figure 3. Comparison of percentage sperm forward progressive motility (FPM) among hormone treatments. Mean ± SD percentage FPM of six hormone treatments, n=27, 6 replicates. ANOVA ($F_{(5,12)}$ = 4.337, p = 0.0174), Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

Osmolality of spermic urine was no different among hormone treatments or between the controls ($p>0.05$). Mean ± SD osmolality (mOsmol/kg) across the treatments, control 1 (ARS) and control 2 (no injection) are shown in Table 3.

Table 3. Osmolality of spermic urine from all hormone treatments and controls, n=27, 6 replicates for hormone treatments and controls.

	GnRH-A 0.1 µg/µl	GnRH-A 0.2 µg/µl	GnRH-A 0.4 µg/µl	hCG 0.5 IU/µl	hCG 1 IU/µl	Amphiplex	Control 1 (ARS)	Control 2 (no injection)
Osmolality (mOsm/kg)	89.23±1.76	84.00±4.36	84.90±4.38	95.67±3.33	83.30±9.77	96.50±7.21	81.61±4.15	84.65±8.53

In contrast, there were significant differences in the spermic urine pH among hormone treatments ($F_{(7,24)}$ = 24.16, p< 0.0001). All the treatments differed from the controls (p<0.0001) and among the treatments, GnRH-A 0.4 μg/μl and hCG 0.5 IU/μl (Mean+SD 7.15 ± 0.07; 7.86 ± 0.05, adjusted p = 0.0074) varied, with the latter showing a higher spermic urine pH. There were no differences among the rest of the treatments or between the controls (p >0.05) (Fig 4).
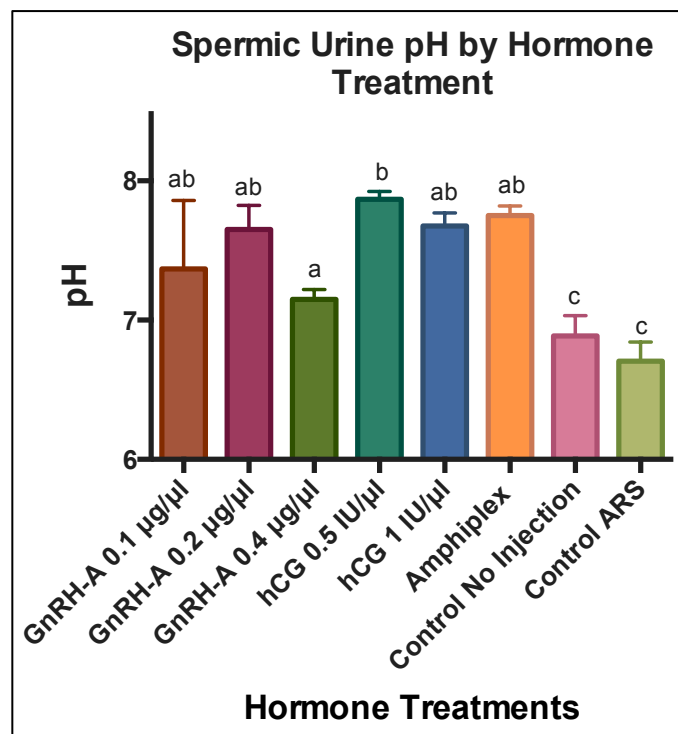
Figure 4. Comparison of spermic urine pH among hormone treatments. Mean ± SD spermic urine pH of six hormone treatments and two controls, n=27, 6 replicates. ANOVA ($F_{(7,24)}$ = 24.16, p < 0.0001), Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

After an assessment of all the treatments in the previous experiments, we selected the best working concentration from each hormone in terms of sperm concentration (GnRH-A 0.4 µg/µl, hCG 1 IU/µl and Amphiplex) for correlation analysis.

For the GnRH-A 0.4 µg/µl treatment, osmolality moderately correlated with motility ($r = 0.4502$, $n = 29$, $p = 0.0110$), FPM ($r = 0.3676$, $n = 29$, $p = 0.0419$) and pH ($r = 0.4134$, $n = 29$, $p = 0.0208$). In addition, pH strongly correlated with motility ($r = 0.5497$, $n = 29$, $p = 0.0014$), FPM ($r = 0.6654$, $n = 29$, $p<0.0001$) and moderatedly correlated with osmolality ($r = 0.4134$, $n = 29$, $p = 0.0208$). For the hCG 1 IU/µl treatment, neither osmolality nor pH correlated with motility, FPM or between them ($p>0.05$). For the Amphiplex treatment, osmolality moderatedly correlated with pH ($r = 0.4259$, $n = 29$, $p = 0.0301$). Aditionally, pH strongly correlated with motility ($r = 0.5312$, $n = 29$, $p = 0.0052$), moderately correlated with FPM ($r = 0.4123$, $n = 29$, $p = 0.0363$) and osmolality ($r = 0.4259$, $n = 29$, $p = 0.0301$) [144]

Morphology

Figures 5, 6 and 7 describe the general morphology of *A. zeteki* spermatozoa. For the specific assessment of morphological features, which includes comparing cells for the presence of normal acrosome, head and tail, there was no evidence of any hormone treatment affecting these characteristics ($p>0.05$). Percentage of normal cells was similar between treatments and the testicular sperm for acrosome and head integrity, while for tail integrity there was no difference among treatments but all of them differed from testicular samples ($p<0.0001$) (Fig 5 and 8).
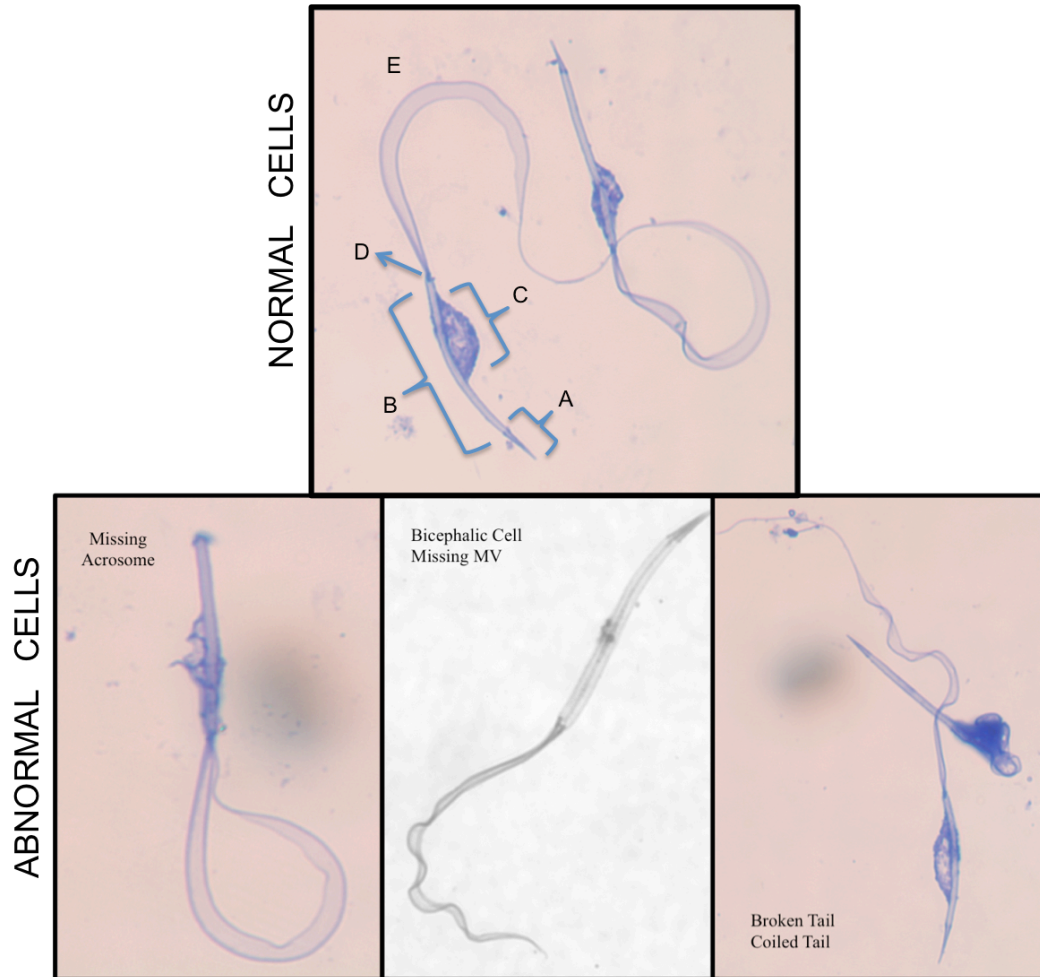
Figure 5. Images of normal vs. abnormal Panamanian Golden Frog (PGF) Spermatozoa stained with Coomassie Blue (Magnification of 1000X). The top picture shows two normal cells with letters indicating the traits that are assessed for morphology analysis: A) Acrosome, B) Head, C) Mitochondrial Vesicle (MV) and E) Tail. D) Midpiece, is not included in the morphological assessment. Average length of the head (including the acrosome) and the tail were 24.53±2.99 μm and 43.38±5.60 μm respectively. On the bottom panel, Left: sperm cell missing the acrosome with a broken MV; Middle: bicephalic cell with missing MV; Right: two cells, one with a broken tail and another with a coiled tail.
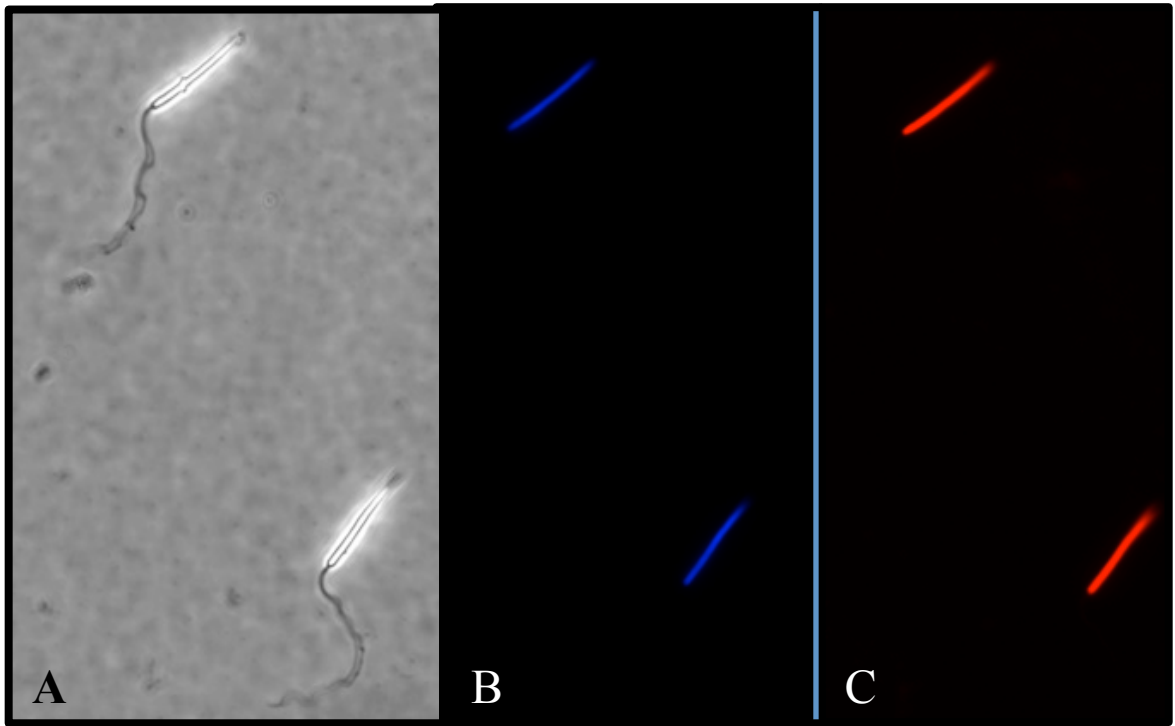
Figure 6. Nucleus of *A. zeteki* spermatozoa (400X magnification). A) Phase contrast B)

Hoechst 33342 Staining and C) Propidium Iodide Staining.
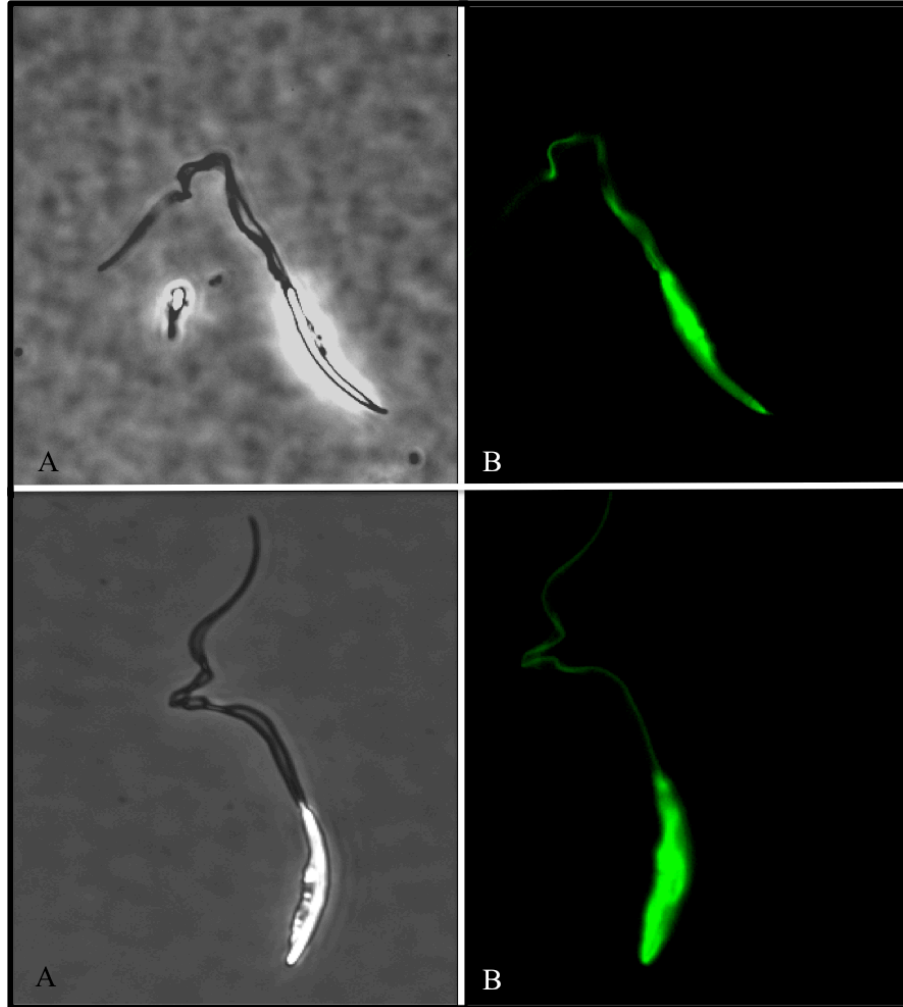
Figure 7. Mitochondrial vesicle of *Atelopus zeteki* spermatozoa (Magnification of 1000X). A) Phase contrast. B) Mito Tracker staining showing respiring mitochondria.
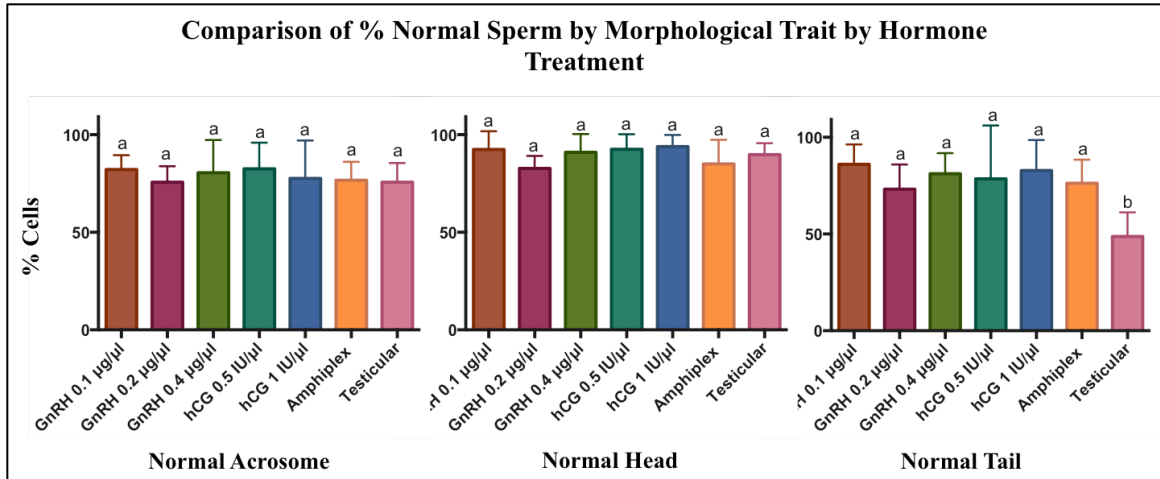
Figure 8. Comparison of normal cell percentage by morphological feature by hormone treatment. Mean ± SD percentage of cells, n = 27. Kruskal-Wallis test for acrosome morphology (H = 4.743, 6 d.f., $p>0.05$), ANOVA for head morphology ($F_{(6, 62)} = 0.6675$, $p>0.05$) and tail morphology ($F_{(6, 62)} = 0.8546$, $p<0.0001$). Tukey's multiple comparison test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

We selected the best working concentration from each treatment (GnRH-A 0.4 µg/µl, hCG 1 IU/µl and Amphiplex) to continue with the DNA integrity and seasonality assessments.

DNA Integrity

The DNA integrity assessment showed no difference in the percentage of intact DNA among the three hormonal treatments or between the treatments and the testicular sperm control ($p>0.05$).
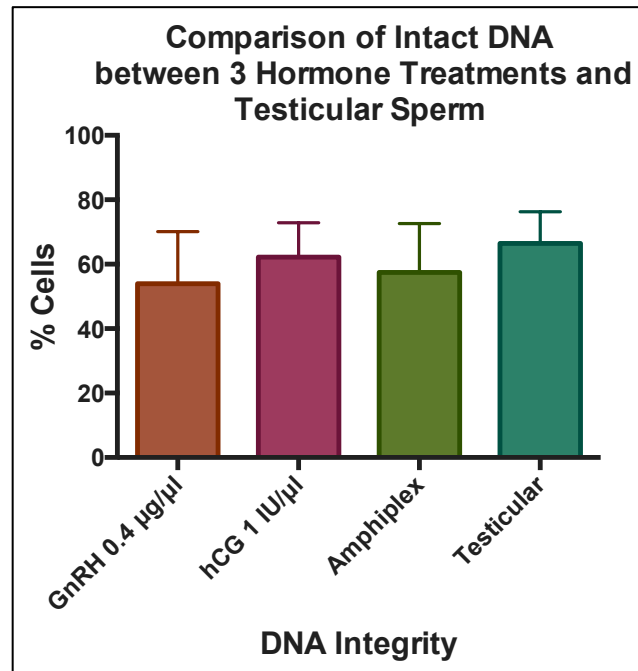
Figure 9. Comparison of percentage intact DNA by hormone treatment. Mean ± SD percentage of cells, n = 18. ANOVA ($F_{(3,25)} = 0.4028$, $p > 0.05$).

Seasonality

Sperm concentration did not differ during the seasons of the year among GnRH-A 0.4 μg/μl, hCG 1 IU/μl and Amphiplex ($p>0.05$) (Fig 10). Neither did osmolality nor pH ($p>0.05$).
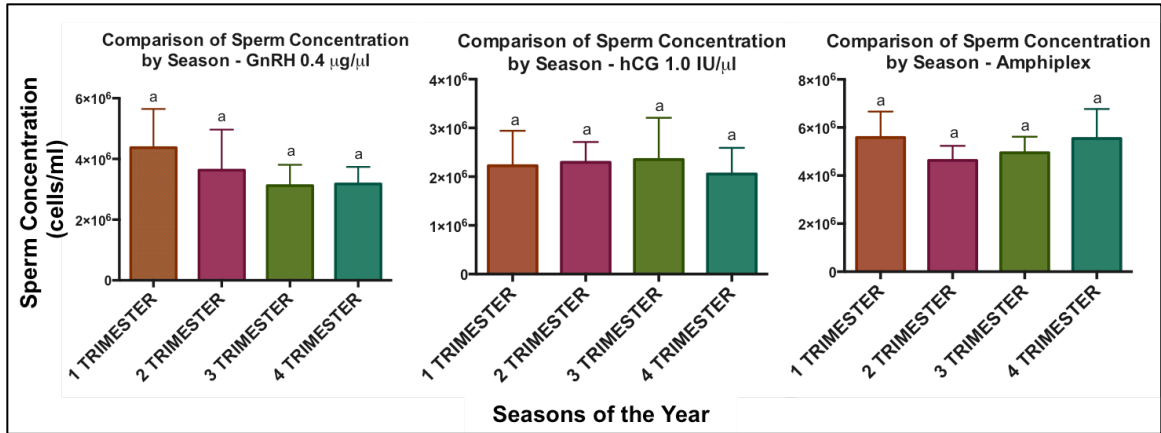
Figure 10. Comparison of sperm concentration during the 4 seasons of the year among GnRH-A 0.4 μg/μl, hCG 1 IU/μl and Amphiplex. Mean ± SD sperm concentration, n = 29. ANOVA for seasons of the year by hormone: GnRH-A 0.4 μg/μl ($F_{(3,25)}$ = 1.15, $p>0.05$), hCG 1 IU/μl ($F_{(3,16)}$ = 1.34, $p>0.05$) and Amphiplex ($F_{(3,39)}$ = 0.64, $p>0.05$), Tukey's post hoc test results are shown as letters above the columns.

Discussion

The induction of spermiation through the use of exogenous hormones, part of the ART toolbox, is a growing trend in the field of amphibian conservation [32,33,52,65,81]. The elaboration of a successful sperm collection protocol for a given species is an intricate process, usually species-specific, that requires measuring, in terms of cell concentration, integrity and viability, the effect of alternatives in the type of hormone to be utilized, concentrations, doses, administration and post-injection collection times, as well as the effect of such treatments in cell functionality. This study assessed the effect of different hormone treatments in *Atelopus zeteki*'s sperm concentration, motility, forward progressive motility, spermic urine osmolality and pH, cell morphology, DNA integrity and seasonality.

Hormone Stimulation and Sperm Collection

Our results showed that all the hormone treatments utilized in this study stimulated spermiation in the male PGF (*A. zeteki*), some of which were more effective than others in terms of sperm concentration during the production peaks. All treatments produced a bell-shaped time-dependent curve, stimulating the production of spermatozoa after 30 min post-injection, with increasing concentrations until reaching a peak at different time points, followed by a constant drop over time, with very low concentrations of spermatozoa collected at 24 h post-injection. This time-dependent response has been observed before using hCG and GnRH-A in other species of Bufonids [120,145]. We were not able to collect samples past 24 h post-injection due to time constraints and accessibility to the holding area. It remains to be determined how much longer, if any, the effect of these hormones lasts on *A. zeteki*, as it has been determined for some species of fish [130].

It is important to understand the effect of different hormones and the concentrations administered to stimulate spermiation and peak production in *A. zeteki* as part of the ART protocols for the species. Future studies will help us understand the time-dependent response in females in order to establish a synchronized spawning and artificial fertilization protocol for this critically endangered species.

The two most efficient treatments for the recovery of high concentrations of spermatozoa were GnRH-A [des-Gly^{10}, D-Ala^{6}, Pro-NHEt^{9}] 0.4 µg/µl (4 µg/gbw) and the hormone cocktail Amphiplex (0.4 µg/gbw of GnRH-A [des-Gly^{10}, D-Ala^{6}, Pro-NHEt^{9}] supplemented with 10 µg of Metoclopramide hydrochloride).

GnRH-A 0.4 µg/µl reached a 1-hour sperm production peak after 2.5 h post-injection, as also observed with GnRH-A stimulation in *Rana temporaria* [120], while Amphiplex reached a 1-hour sperm production peak after 3.5 h post-injection, with GnRH-A 0.4 µg/µl eliciting a more rapid response (Table 2). Nevertheless, Amphiplex stimulated the production of signifantly higher concentrations of spermatozoa having 10 times less the concentration of GnRH-A 0.4 µg/µl . This can be explained by the fact that GnRH-A 0.4 µg/µl produced an average concentration of $2.6x10^6 \pm 2.12x10^5$ cells/ml at its peak followed by a constant decrease, while Amphiplex produced the same average concentration at 2.5 h post-injection (GnRH-A 0.4 µg/µl's production peak) and instead of decreasing, it kept increasing until reaching the 3.5 h peak with an average concentration of $4.45x10^6 \pm 7.1x10^4$ cells/ml, suggesting that metoclopramide potentiated the effect of the lower GnRH-A concentration present in Amphiplex.

As stated before, species responses to hormonal stimulation might vary due to species specificity [120]. A study by Kouba et al., 2012, in *Anaxyrus americanus* showed no clear peak of sperm production between 3 and 24 hrs post-injection of GnRH-A, while hCG stimulation produced a peak between 3 and 9 hrs post-injection. Our results show a clear stimulation peak for all the treatments indicating that this species responds similarly to the different hormones and concentrations used in this study.

Administration of GnRH-A at 0.1 and 0.2 µg/µl succesfully induced the production of spermatozoa while yielding lower concentrations ($\sim 1.6x10^6$ cells/ml) than those obtained with GnRH-A 0.4 µg/µl, which seems to be the best GnRH-A working concentration from the ones we utilized for the collection of spermatozoa in *A. zeteki*. Even though higher concentrations of GnRH-A have been successfully used in other

species, we chose these particular concentations because: 1) these frogs are small, with weight ranging anywhere from 2.5 to 4.5 g in average and doses are usually weight dependent; 2) we did not want to risk over-dosing these critically endangered specimens; 3) a prior pilot study yielded positive results using GnRH-A 0.2 μg/μl, reason why we decided to try two more concentrations, one lower and one higher than 0.2 μg/μl; 4) we chose 0.4 μg/μl as the concentration above 0.2 μg/μl because we wanted to test 10 times the concentration of GnRH-A present in Amphiplex to assess the effect of metoclopramide in the spermiation response of the species. It remains to be verified if higher concentrations of GnRH-A produce a similar or better response than the one obtained with GnRH-A 0.4 μg/μl.

The positive response of *A. zeteki* to stimulation with GnRH-A might be explained because this analog of the mammalian GnRH acts on the brain, promoting a broader and more complete physiological response [74]. GnRH-A exhibits high affinity for the type 1 GnRH-R, which are the most abundant GnRH receptors in the anterior pituitary of anurans [64]. Furthermore, this analog also shows longer half-life, higher resistance to degradation and has successfully promoted spawning in other species of amphibians by stimulating the production of endogenous gonadotropins, specially LH, a consequence of the modified alanine in position 6 [30,62,64,66,74,146-150].

While GnRH-A acts on the brain, hCG acts directly on the gonads, since this hormone binds to the same receptors as LH [74,108,120,125,126,151]. There are several reports on the high variability of spermiation responses from several species of amphibians to hCG stimulation [30,52,66,81,108], usually with fewer species showing a positive response to this hormone than to GnRH-A, probably due to the lower affinity of the hormone to the

anuran LH receptor [64,117,120,152] or to an immune response of non-mammalian species due to the bigger size of the molecule [74].

Human Chorionic Gonadotropin induced spermiation at a concentration of 0.5 and 1 IU/μl; nevertheless, concentrations below 0.5 IU/μl failed to induce spermiation in *A. zeteki* (0.125 and 0.25 IU/μl – 0 cells/ml, data not shown), maybe because stimulation with very low concentrations of LH or hCG have been associated with a lack of response from Leydig cells [153].

Our study also revealed that both working hCG treatments stimulated the production of similar concentrations of spermatozoa [$1.57 \times 10^6 \pm 2.1 \times 10^5$ cells/ml (hCG 0.5 IU/μl) and $1.58 \times 10^6 \pm 2.62 \times 10^5$ cells/ml (hCG 1 IU/μl)]. This might be related to *A. zeteki*'s testicular size, receptor sensitivity and reactivity to hCG and/or the number of receptors present in the membrane of Leydig cells, all of which remain to be understood for the species. All these factors might influence the response of *A. zeteki*' Leydig cells to specific concentrations of this hormone where receptor availability and saturation could be playing a part. Another possibility is the existence of negative regulation since LH receptor numbers decrease as LH/hCG concentrations increase, ultimately rendering the cells desensitized or refractory for the production of testosterone, as it has been shown for insulin, thyrotropin releasing hormone and catecholamines receptors [153-155]. A study by Payne et al., in rat Leydig cells revealed that loss of LH receptors is not directly related to a reduction or loss of testosterone production when repeated lower dose injections were applied; in contrast, a marked reduction was observed when a one time high non-physiological dose injection was used. In our study, we utilized a one-time injection of two hCG concentrations that might be high for the species and thus,

generating a refractory response in *A. zeteki*'s Leydig cells. It remains to be verified if applying two consecutive doses of hCG 0.5 IU/µl instead of one hCG 1.0 IU/µl dose would generate a different response in sperm production, or if hCG 0.5 IU/µl is sufficient to produce a maximal response in this species. We did not try higher concentrations of hCG because we currently lack data on the testicular size for the species, we did not want to risk over-dosing the specimens nor generate an immune response to the hormone. Higher concentrations of hCG remain to be tested.

Stress is related with the stimulation of dopamine (DA) neurons and therefore, the production of DA which has been shown to affect or even inhibit reproduction [30,117,118,156,157]. Species kept in captivity are exposed to several internal or external stressors such as nutritional deficiencies, disease, handling, excess or lack of specific environmental cues and crowded enclosures, among others [30,156,158], what suggests that stress might be playing an important role in the impairment of reproduction in some captive species.

There is evidence that DA is an inhibitor of gonadotropin production at the pituitary level on several species of amphibians, freshwater fish, birds and mammals [62,64,66,74,118,128,159]. The effect of DA as a Gonadotropin Release Inhibitory Factor (GRIF) is related to a marked decrease in the LH production, possibly due to an inhibitory effect on the GnRH receptors located in the anterior pituitary, resulting in the final inhibition of ovulation and spermiation [74,118,128,130].

Treatment with Amphiplex stimulated the production of higher concentration of spermatozoa in *A. zeteki* ($\sim 4.45 \times 10^6$ cells/ml) than any other treatment. It is worth to mention that Amphiplex contains 10 times less the concentration of GnRH-A that we

used on the best working GnRH-A treatment alone, plus 10 μg of Metoclopramide, a dopamine (DA) antagonist. Metoclopramide might be blocking or attenuating the inhibitory effect of DA in *A. zeteki*, or increasing the GnRH-A binding affinity to the receptors, as it has been observed with pimozide and domperidone in fish [160], aiding in the production of higher concentrations of spermatozoa due to the stimulation of a LH surge, as it has been observed in other species of amphibians [30,64,66,74,118,128-130].

Sperm motility in amphibians is believed to be regulated by osmotic changes in the extracellular environment as it has been observed in fish as well [69,71,76]. Other factors such as pH and ionic composition of the extracellular environment have been shown to play an important role in the regulation of sperm motility in marine invertebrates, amphibians, fish and mammals [57,71,73,75,76,161-163].

According to our results, none of the hormone treatments seem to be detrimental for the motility of *A. zeteki* spermatozoa during peak production, although some differences were observed. Percentage motility was higher than 85% for all the hormone treatments with GnRH-A 0.4 μg/μl showing the lowest percentage (87.25%) at its peak compared to the rest of the treatments (all others > 90%). Although GnRH-A 0.4 μg/μl showed significantly lower motility than hCG 1 IU/μl only, it also exhibited lower motility trending towards significance than two other treatments (GnRH-A 0.1 μg/μl, *p*=0.06; Amphiplex, *p*=0.052). It is worth to mention that, while not significantly, percentage motility decreased while GnRH-A concentrations increased. hCG 0.5 IU/μl, hCG 1 IU/μl and Amphiplex exhibited similar percentage motility during the production peaks. As we were not able to collect any spermic urine without hormonal stimulation, comparison of sperm motility between hormonally and non-hormonally stimulated

spermic urine samples was not possible, however, non-hormonally stimulated testicular sperm samples from 11 individuals showed high percentage motility (>90%) when activated with water (data not shown).

When trying to explain why spermatozoa obtained after stimulation with GnRH-A 0.4 μg/μl showed the lowest percentage of motility, we assessed spermic urine omsolality from all the treatmetns but found no differences among hormones nor between hormones and controls, suggesting that neither hormone treatment affected the osmolality of the spermic urine in *A. zeteki* nor osmolality had an effect on the lower percentage of motility from the GnRH-A 0.4 μg/μl treatment.

Nevertheless, pH did significanlty differ among treatments and between all treatments and the controls, suggesting that the hormone treatments used in the study increased *A. zeteki*'s spermic urine pH. Several studies in fish have established a relationship between an icrease in sperm pH and hormone treatments. A study by Clearwater and Crim in yellowtail flounder sperm using different long-term GnRH-A delivery systemas revealed an increase in milt pH and motility compared to non-treated sperm. A study by Hajirezaee et al., in caspian brown trout sperm showed an increase on milt pH after treatment with a single dose injection of GnRH-A. Similarly, Seifi et al., determined that treatment with ovaprim (a cocktail of salmon GnRH-A+domperidone, a DA antagonist) increased milt pH in carp.

Several studies on fish spermatozoa have suggested the role of 17α,20β-DP (17α,20β,dihydroxy-4-pregnen-3-one), a progestogen involved in increasing the fish sperm duct pH, milt pH and hydration, on the adquisition and enhancement of sperm motility [116,164-166]. Kobayashi et al., 2013, suggested a function of an isomer, 17α,20α-

DP, on the induction of spermiation in *Rana nigromaculata*, with no mention of its function on the activation of amphibian sperm motility [166]. More research involving the impact of hormonal stimulation on the production and function of 17α,20β-DP or 17α,20α-DP in amphibian sperm motility and pH could shed some light on the specific effects of hormonal stimulation in this class.

Even though all the treatments increased the spermic urine's pH, treatment with GnRH-A 0.4 μg/μl showed significantly lower pH than hCG 0.5 IU/μl and also exhibited lower pH trending towards significance than GnRH-A 0.2 μg/μl (p=0.087), hCG 1.0 IU/μl (p=0.063) and Amphiplex (p=0.068). Alkaline pH has been shown to have a positive effect on the sperm motility of some species of fish [76,132,163,167]. A study by Christensen et al., showed that osmolality and pH together impact *Xenopus laevis* sperm motility. Another study by Takai et al., in marine and freshwater fish sperm motility revealed that sperm extracellular pH is equivalent to the intracellular pH. If these conditions apply to *A. zeteki* spermatozoa, we could infer that treatment with GnRH-A 0.4 μg/μl, even thought it significantly increased the extracellular pH compared to the controls, the increase was not as high as compared to the other treatments. This might have decreased the intracellular pH of the spermatozoa, acidifying the cytoplasm, which, in turn acidified the mitochondrion, an alkaline organelle responsible for producing the necessary energy for motility. Furthermore, sperm cytoplasm acidification has been previously associated with the partial or total inactivation of the dynein protein, responsible for the movement of the sperm tail [76,162,168,169]. Even though GnRH-A 0.4 μg/μl produced high concentrations of spermatozoa with good percentage motility, this treatment altered the pH of the spermic urine and thus lowered the motility in *A. zeteki*

spermatozoa, effect that seems to be counterbalanced by lower GnRH-A concentrations, metoclopramide in the Amphiplex treatment and absent in the hCG treatment.

FPM also showed significant differences among treatments, with GnRH-A 0.2 µg/µl showing lower FPM than hCG 1.0 IU/µl. Intriguingly, treatment with hCG 1.0 IU/µl showed higher FPM, although not significant, compared to most of the treatments. The specific mechanism by which hormonal stimulation might affect sperm forward progressive motility is not yet understood. Nevertheless, there is evidence that higher doses of hCG increase FPM in amphibians. A study by Kouba et al., 2012, showed that treatment with 300 IU of hCG in American toad spermatozoa produced the highest FPM compared to lower doses of hCG. In our study, higher doses of hCG produced the highest percentages of FPM, suggesting a boosting dose-dependent effect of this hormone on the FPM of *A. zeteki* spermatozoa. Further research is needed to understand the effect of hCG stimulation on the constitution of seminal plasma and its effect on *A. zeteki* sperm motility and FPM as it has been suggested for the Japanese eel, *Anguilla japonica* [170].

Christensen et al., reported that osmolality and pH impact sperm motility in *X. laevis*. Even though they did not observe a significant association between pH and motility, there was an increase in sperm motility at pH 7.0 compared to solutions with lower or higher pH. They concluded that there is a species-specific pH window for optimal sperm motility that will not inactivate ATPases, therefore, potentiating motility. Similarly, their results showed that medium osmolality impacts motility and FPM, with lower and higher dilutions show lower motility and FPM.

It is important to mention that the study by Christensen et al., 2004, utilized testicular sperm while ours used hormonally induced spermic urine where hormones

might play a role modifying the composition of the extracellular environment. Based on the results of our study, osmolality and pH of spermic urine are associated to each other and both influence sperm motility and FPM in the GnRH-A 0.4 μg/μl and Amphiplex treatments. Nonetheless, pH seemed to have a stronger effect than osmolality in the percentage motility and FPM of *A. zeteki* spermatozoa. A reasonable explanation might be that, even though there were slight variations, the osmolality of the spermic urine was similar among hormonal treatments and we did not assess the effect of different osmolalities in *A. zeteki* spermatozoa, which could have resulted in a stronger correlation. As shown by the reults above, all hormone tratments increased the spermic urine pH compared to the controls (plain urine) and sperm motility and FPM showed a stronger association with spermic urine pH just in treatments conatining GnRH 0.4 μg/μl. This could indicate that stimulation with GnRH 0.4 μg/μl might be changing the composition of *A. zeteki* spermic urine in a way that impacts sperm motility and FPM.

Morphology

Our assessment of *A. zeteki* sperm morphology suggests the presence of an elongated filiform head with an acrosomal complex on the anterior portion, followed by a small midpiece at the posterior end of the head. The tail is almost twice the length of the head and clearly shows two filaments joined by an undulating membrane, ending in a single filament at the posterior end of the tail. Siqueira et al., published the only available sperm ultrastructure study on an *Atelopus* species. Their results revealed that *A. spumarius* spermatozoa have an average head size of 19 μm and a tail of 40 μm [171]. Our results suggest that the head of *A.zeteki* is slightly bigger than that of *A. spumarius*, while the tail length is similar.

The presence of an undulating membrane and auxiliary fibers in the sperm tail of some species of anurans has been associated with phylogeny, particularly with the lineage of Bufonids, and with the fertilization mode of the species, with some of the species with aquatic external fertilization presenting a tail with these structures [45,131,171]. The only report that we could find on the reproductive behavior of *A. zeteki* indicates that females oviposit and attach the eggs in the rocks of streams with rapid currents during the dry season, when the volume of water is low [29]. The presence of an undulating membrane in the tail of *A. zeteki* spermatozoa may be related to the need of the cells to reach the eggs against rapid water currents and to penetrate the jelly coat that surround the egg to achieve fertilization [78,131].

After nucleic acid staining, we were able to observe that the nucleus is a long, conical structure that occupies most of the head of *A. zeteki* spermatozoa. A similar nuclear morphology has been observed in other species of anurans [47,48,171,172].

All the cells collected during the study showed the presence of a structure that has been described before in Bufonid spermatozoa and associated with sperm motility: the Mitochondrial Vesicle (MV) [48,55,61]. Obringer et al., described the structure as a residual cytoplasmic droplet in the head of American and Wyoming toad spermatozoa. Waggener and Carroll referred to it as an accessory cell attached to the head of *Lepidobatrachus laevis* and suggested a role in sperm motility.

The MV is a cytoplasmic droplet-like structure that contains a high concentration of moving mitochondria, and is located in the external mid to lower part of the sperm head [28,47,55,61]. The MV (Figure 5 and 7) is a very fragile structure. Exposure to osmotic and temperature changes, as well as to mechanical stress (maceration and centrifugation),

might cause the loss or damage of the MV [61]. The presence of such structure makes sperm manipulation in *A. zeteki* even more challenging. Understanding the role of this structure in the functionality, activation and viability of the cells in future studies might provide valuable information on the physiology of the cells.

Our evaluations on the morphology of *A. zeteki* spermatozoa are based on the use of different staining techniques. An ultrastructural assessment with the use of Transmission Electron Microscopy is a more precise method that would provide specific details associated to the physiology and functionality of these cells.

Sperm morphology is an indicator of cell viability and fertilization potential [81]. To our knowledge, this is the first study on *A. zeteki* sperm morphology. Our data suggest that more than 73% of the cells present a normal morphology and that none of the hormonal treatments affected the morphology of the cells. Since we could not collect any sperm samples without hormonal stimulation as controls, we compared the morphology of the hormonally stimulated samples with the testicular sperm samples obtained from the 11 euthanized males. Acrosome and head morphology did not differ between hormonally stimulated and testicular samples nor among hormone treatments, nevertheless, testicular sperm tail morphology significantly differed from all hormone treatments, showing a lower percentage of normal tails. One explanation for this difference could be that spermic urine contains strong, activated mature spermatozoa that have been ejaculated after hormonal stimulation. Testicular sperm samples are collected by maceration of the testes, where a mixture of mature and immature cells is obtained. The fragility of immature cells combined with the mechanical damage applied by maceration could have

caused a higher percentage of damage in the testicular sperm tails, given that the most observed tail abnormality for these samples was "broken tail" (data not shown).

We selected the best working concentration from each treatment to compare the effect of the hormones in *A. zeteki* sperm DNA integrity and seasonality.

DNA integrity

Our results showed that none of the treatments induced DNA fragmentation in the nucleus of *A. zeteki* spermatozoa, suggesting that neither is detrimental for the viability of the cells nor for the fertilization potential compared to the testicular sample.

To our knowledge, there are an extremely limited number of studies assessing sperm DNA integrity in amphibians [134,173]. A study by Pollock et al., on the percentage of sperm DNA integrity in *X. laevis* spermatozoa validated the effectiveness of the modified Sperm Chromatin Dispersion test (SCD) and compared it against two other techniques: In situ Nick Translation (ISNT) and double Comet Assay, concluding that SCD is an effective technique to assess sperm DNA integrity in this species. They reported a maximum of 61% of sperm DNA fragmentation using this technique in 3 males *X. laevis*.

In our study the overall percentage of fragmented DNA across treatments, including testicular sperm, was 40%, employing a *n*=34. We utilized the Terminal Deoxynucleotidyl Transferase dUTP Nick end-Labeling (TUNEL) technique because this is one of the most commonly used techniques for evaluation of sperm DNA integrity in humans and other species [174-177]. This technique, as well as the Comet Assay, detects the presence of single and double-stranded breaks in the DNA, while others measure the DNA denaturation potential, which can underestimate the amount of DNA damage in a cell [137,177,178].

A study by Mitchell et al., revealed that the TUNEL assay underrates the amount of damaged sperm DNA in humans and rats when comparing the traditional TUNEL technique to a modified one using dithiothreitol (DTT), a reagent that relaxes the chromatin to allow access to the broken DNA strands. Relaxation of sperm chromatin may be necessary for a better assessment of DNA integrity when dealing with mammalian sperm since chromatin is tightly packaged around protamines in the nucleus [134,135,137,173,174,177,179]. However, there is evidence that fish and anuran sperm chromatin, depending on the genus, contains protamine-like proteins and/or a higher number of histones in its packaging, suggesting a different level of chromatin condensation than that of mammalian sperm chromatin [135,173]. This suggests that the percentage of broken DNA detected in these species might be more accurate than the one detected on mammalian sperm DNA when utilizing this technique.

Most of the studies on human sperm DNA fragmentation agree that the cutoff for infertile men is >20% [137,175,179-181]. Our results in *A. zeteki* double the value of the human sperm DNA integrity threshold. Since there is no established cutoff for amphibians, we speculate that some of the reasons for what it seems a high percentage of fragmented DNA in this species could be: 1) chromatin is highly compacted inside the sperm nucleus where topoisomerases might relieve torsional stress by inducing nicks in the DNA [135,179]; 2) amphibian sperm activates after a drop in the osmolality of the extracellular environment, which causes an osmotic shock that might induce DNA fragmentation [69,76,134]; 3) sperm manipulation can increase the DNA susceptibility for damage [182,183]; 4) the TUNEL assay does not discriminate between viable or dead cells, suggesting that the overall DNA fragmentation value could include non-viable cells [174,176].

DNA integrity is an important factor to consider and understand when developing ART protocols for a species, especially Intracytoplasmic Sperm Injection (ICSI). Bypassing the natural selection of gametes before and during fertilization by injecting a genetically damaged sperm directly into an oocyte might increase the probability of producing non-viable offspring or passing down non-desirable genetic traits to the next generation [133,137,184,185].

Seasonality

Amphibians, specially anurans, exhibit a wide variety of reproductive patterns [186,187]. For tropical species that breed near or in the water, seasonality is usually defined by rainfall in regions where there are discernible dry and rainy seasons [187]. Species that oviposit in streams usually breed during the dry season, when the water level is lower [186,187]. To our knowledge, the only report on the reproductive ecology of *A. zeteki* suggests the species breeds from late November to January, during the start of the dry season [29]. We found no variation in the production of spermatozoa, spermic urine osmolality or pH over the year, which suggests that the hormone treatments we utilized successfully promote spermiation in *A. zeteki* year round and that spermic urine osmolality and pH are not altered throughout the year. Future studies on the reproductive physiology of female *A. zeteki* might shed more light on the effect of hormonal stimulation in the reproductive seasonality of the species.

This study was significant for producing important, otherwise missing information on the reproductive biology of the male *A. zeteki* and for the design of a safe and repeatable sperm collection protocol for the species. The knowledge gathered during the research will help us develop and implement other ART's such as sperm

cryopreservation and AF, techniques that mitigate the reproductive problems amphibian species face in captivity. Moreover, the protocols and techniques used in this study can now be tested on other endangered amphibian species in captivity, kept in Panamanian amphibian conservation initiatives such as El Valle Amphibian Conservation Center (EVAAC) and the Panama Amphibian Rescue and Conservation Project (PARC).

After analyzing our results, we recommend the use of Amphiplex, via intraperitoneal injection, for the collection of highly concentrated *A. zeteki* sperm samples. We also recommend collecting the sperm samples 3.5 h post-injection. Repeatability of the sperm collection protocol was confirmed since all of the individuals in the colony were collected several times, year round, for the past five years.

Chapter 3: <u>Effect of three different extracellular conditions on the motility and viability of Panamanian Golden Frog (*Atelopus zeteki*) spermatozoa.</u>

Abstract

Sperm motility is essential for the cells to reach the eggs and achieve fertilization, and it is the most commonly used parameter to assess sperm quality and viability. Amphibian spermatozoa are very sensitive to extracellular conditions since motility activation depends on the osmolality of the external environment. The accurate implementation of ARTs depends on the optimal handling of gametes and understanding what conditions are detrimental for their viability. Our aim was to determine the effect of three extracellular conditions (raw spermic urine kept at room temperature, raw spermic urine kept at 4 ºC and water diluted spermic urine) on the motility parameters, morphology and DNA integrity of hormonally induced *Atelopus zeteki* spermatozoa. We collected sperm samples after hormonal stimulation and all parameters were assessed (concentration, osmolality, pH and volume). Our results revealed that *A. zeteki* spermatozoa maintained high percentage motility and FPM when kept in spermic urine at room temperature and at 4 ºC for at least 46 min after collection, with no detrimental effect of hormonal stimulation. Dilution in water greatly reduced sperm motility parameters, mainly FPM, DNA and morphological integrity of *A. zeteki* spermatozoa.

Introduction

Motility is considered a common and important sperm quality indicator since it involves the normal development and correct functioning of several cellular systems working together to achieve motion, forward progression and finally, fertilization [140,167]. Among such systems are: the plasma membrane, responsible for mediating the activation responses to external conditions, mitochondria, responsible for energy storage and use, and axoneme, responsible for flagellar movement [182]. Sperm motility is absolutely necessary for the sperm to reach and penetrate the eggs [72,76,140,188].

In amphibians with external fertilization, as in freshwater fish, sperm motility activation is closely associated with the osmolality of the extracellular environment as sperm is immotile (inactive) inside the testis where the osmolality reaches 300 mOsm/kg and activated upon contact with lower osmolalities [55,71,73,88,167,182,189-192]. Additional factors such as HCO_3^-, K^+, Na^+ and Ca^{2+} concentrations have been shown to be critical for the activation of sperm motility in some species of fish such as trout, carp, sturgeon and the Japanese eel [182,193,89,170,189,190].

Activation of sperm motility has been also associated with other critical elements such as changes on the plasma membrane potential and lipid composition, extracellular and intracellular pH and ion concentration, and the temperature of the extracellular environment [76,167,189,194,195]. Furthermore, a signaling pathway involving increasing concentrations of intracellular cyclic adenosine monophosphate (cAMP) and Ca^{2+}, with the subsequent phosphorylation of specific proteins by cAMP-dependent protein kinases, has been associated with the sperm activation process in some species of mammals, including humans; in salmonids and sea urchins [77,89,90,194]. Nevertheless, for some species

of fish such as carp, sea bass and striped bass, and in mammals like bulls, the activation of sperm motility is independent of the cAMP pathway [73,91].

The mechanisms behind amphibian sperm motility activation are not as well understood as they are in fish. The spermatozoa's plasma membrane composition of several species of fish, specially trout and carp, has been deeply studied, exhibiting the presence of ionic channels and receptors directly involved in the activation of motility [182,196,197]. The low membrane permeability of trout sperm to extracellular glucose has been associated to the incapacity of these cells to utilize it as an external energy source to sustain motility for long periods of time, depending only on the sperm ATP stores to activate the dynein ATPase and to initiate and maintain flagellar beating [182,198].

In salmonids and sturgeons, initiation of motility has been linked to changes in potassium concentration in the seminal plasma or that of the extracellular environment, while in cyprinids and marine fishes; activation of motility is triggered by changes in osmolality [182,190,194,195,198,199]. Nevertheless, ions such as Na^+, K^+, Ca^{2+} and Cl^-, are known to influence the initiation of sperm motility in all these groups of fish either by affecting their own intracellular concentrations or by causing changes in the osmolality of the fertilization environment [73,75,88,192,194,197].

Studies in rainbow trout have revealed that activation of salmonid sperm motility depends on the maturation stage of the cells as well as on the extracellular $[K^+]$ [188,190,198]. Dilution of extracellular K^+ triggers a K^+ efflux, causing a change in the membrane potential which promotes a Ca^{2+} influx and activates the intracellular cyclic adenosine monophosphate (cAMP) molecular cascade [88,89,190,194,196].

Contrary to what is observed in salmonids, extracellular $[K^+]$ is not the main trigger for sperm motility activation in freshwater fish, marine fish and amphibians, where fluctuations in intracellular ion concentrations appear to be caused by the expansion or contraction of the cells in response to the osmolality of the extracellular environment [57,71,194,196,197,200]. Intracellular $[K^+]$, $[Na^+]$ and pH_i are believed to play a role further down the activation pathway after a change in osmolality initiates motility in freshwater and marine fish [75].

A rearrangement of the lipid content and a change in the permeability of the plasma membrane caused by the osmotic shock are believed to be associated with this kind of sperm motility activation [73]. Research by Perchec Poupard et al., 1997, showed that carp spermatozoa motility activation is triggered by a change in the external osmolality, independently from the membrane potential or ion concentrations, followed by an increase in head volume, mediated by the passive diffusion of water. This study also suggested that, contrary to what happens in erythrocytes and bacteria [75], water movement in carp spermatozoa is not auto-regulated, depending only on the extracellular solute concentration. Interestingly, another study by Krasznai et al., 2000 suggested a role of membrane polarization in carp sperm motility activation. According to their results, the decrease in the extracellular osmolality is the initial trigger for sperm activation, followed by a change in extracellular $[K^+]$, causing a hyperpolarization of the membrane, producing a Ca^{2+} influx and initiating motility.

Research by Bernardini et al., 1988 showed that testicular sperm from *Xenopus laevis* becomes activated upon contact with hypo-osmotic media and remains quiescent in media of high osmotic strength, independently of $[K^+]$, $[Na^+]$ or $[Ca^{2+}]$. The group also

suggested that Ca^{2+} and pH_i are not related to sperm activation in this species. Similarly, a study by O'Brien et al., 2011 revealed that the activation of *Bufo arenarum* spermatozoa is triggered by the dilution of the fertilization media regardless of the concentrations of ions or the presence of egg water [a solution obtained by incubation of oocytes in distilled water thought to stimulate or enhance sperm motility [200]], and that the process is mediated via the cAMP/PKA signaling pathway through membrane osmo-sensing proteins, transmembrane adenylate cyclase and G-protein activity.

Duration of sperm motility varies greatly among species and is influenced by several factors such as the temperature, pH_o and dilution rate of the extracellular environment [32,167,188,201-203]. Several studies in fish suggest that increasing temperatures are directly related to a steady decrease in sperm motility and forward progression [167]. In salmonids and sturgeons, duration of sperm motility is very short, lasting from seconds to less than 5 minutes in higher and lower temperatures, respectively [167,201,204]. In freshwater fish, motility lasts from seconds to minutes as well and temperature has also been associated with duration of sperm motility, with higher temperatures having a marked negative effect on the time the cells remain motile and on the flagellar beat frequency [198,205-207]. Nevertheless, spermatozoa from the ocean pout can maintain motility for 24 h when kept in a mixture of seminal plasma and ovarian fluid at 4 °C [188]. The negative effect of too low or too high temperatures on the duration of sperm motility has been associated with a faster depletion of the cell's energy storage and a change in enzymatic activity [206,208].

In anurans with external fertilization, like *X. laevis*, spermatozoa remain motile for longer periods of time compared to fish sperm [32,57,76,188,209]. A study by Waggener and

Carroll, 1998, revealed that spermatozoa from *Lepidobatrachus laevis* remain active up to 138 minutes before losing motility at 28 °C. In *Rana temporaria*, spermatozoa stored at ~0 °C are motile for up to 6 h, when kept at 4 °C motility persists steadily for 60+ min, while both, motility and forward progression cease completely within 45 min at 22 °C [57]. Research by Browne at al, 2001 and 2002, showed that testicular sperm from *Bufo marinus* retains motility for 10 days when stored at ~0 °C and for 5 days when kept at 4 °C, while testicular sperm from *Limnodynastes peroni* and *Pseudophryne bibroni* retain >10% motility when stored at 0 °C. Similarly, a study by Shishova et al., 2013 demonstrated that testicular spermatozoa from *Rana temporaria* carcasses maintain motility for up to 10 days when kept at 4 °C.

Hormonal stimulation impacts sperm motility in several species. In the Japanese eel, weekly injections of hCG improve sperm motility by the 9th week of treatment [210]. A study by Clearwater et al., 1998 in yellowtail flounder spermatozoa showed that stimulation with GnRH-A increased the percentage of motile cells and their swimming time from an average of 11 sec to 55 sec after stimulation. Similarly, research by Hajirezaee et al., 2011 in Caspian brown trout spermatozoa revealed that treatment with GnRHa increased sperm motility duration in the species. On the other hand, even though there is no specific mention on the duration of sperm motility, a study in crucian carp (*Carassius carassius*) spermatozoa showed that stimulation with Ovaprim® [(D-Arg^6, Pro^{9}NEt)-sGnRH + domperidone] increased sperm concentration while there was no difference in sperm motility or forward progressive motility between that treatment and stimulation with Ovopel® [(D-Ala^6, Pro^{9}NEt)-mGnRH + metoclopramide] [211]. These two commercial preparations were also used to analyze European smelt (*Osmerus eperlanus*)

spermatozoa motility duration where none of the treatments increased nor decreased the average sperm swimming time of around 30 sec in this species [212].

Even though we are not aware of a comparative report on hormones and their impact on the duration of motility, there are some studies that have assessed this metric in hormonally stimulated amphibians. In the European common frog (*Rana temporaria*), spermatozoa obtained after stimulation with hCG remains motile for up to 45 min at room temperature [57]. Using the same hormone, Inoda and Morisawa, 1987, obtained sperm from *X. laevis* and determined that water diluted sperm maintained motility for 10 min after activation. Similarly, Waggener and Carroll, 1998, determined that spermatozoa from *Lepidobatrachus laevis* stimulated with GnRH retained motility for an approximate of 80 min. Another study on *Bufo americanus* spermic urine obtained after stimulation with GnRHa, revealed that the average sperm swimming time does not exceed 30 min [81].

Besides being the main trigger for activation of freshwater fish and amphibian sperm motility, osmolality plays an important role in the duration of motion as well [32,76]. Even though amphibian sperm is more resistant than mammalian sperm to hypo-osmotic shock, exposure to low osmotic strengths for long periods of time results in the disruption of the plasma membrane, swelling of the flagellum and mitochondria and finally, cell death [65,69,73,75,76,213,214]. In the wild, the initial velocity of amphibian sperm right after contact with the fertilization media is thought to be more important than duration itself since the eggs become swollen and impenetrable minutes after contact with water [11,69,76]. In cane toad (*Bufo marinus*) spermatozoa, motility can last from 1 h at 5mOsm/kg to 5 h at 40 mOsm/kg [32]. Sperm from *X. laevis* steadily loses motility if diluted below 40 mM NaCl, only remaining motile for up to 3 min [60]. Spermatozoa from *Rana sylvatica*

exposed to pond water osmolality (~5 mOsm/kg) remain motile for 10 min before osmolysis [69].

Hypo-osmotic shock has been related to a rearrangement of the lipid domains in the sperm plasma membrane with subsequent swelling and disruption of the cells [215,216]. A study by Marian et al., 1993, in carp spermatozoa determined that dilution of the cells in either distilled or fresh water rendered the cells completely permeable to PI. Other studies have demonstrated the complete rupture of trout and guppy spermatozoa when exposed to low osmolalities, resulting in cell death [217].

Osmotic shock can cause damage in other cell compartments [218]. Exposure to hypo-osmotic environments has been related to an increase in the production of Reactive Oxygen Species (ROS) in skeletal muscle, mouse fibroblasts, horse and rhesus macaque spermatozoa [216,218-220]. A study by McCarthy et al., 2010, determined that exposure of rhesus macaque spermatozoa to hypo-osmotic environments increases the O_2^- concentration in the cells accompanied with increased membrane lipid peroxidation and a marked decrease in total motility and forward progressive motion. Burnaugh et al., 2010 found that horse spermatozoa exposed to extreme hyper or hypo-osmotic conditions showed an increase production of superoxide leading to an increasing amounts of ROS, which might lead to cytotoxicity and cell damage.

Moderate amounts of ROS in spermatozoa, as in most cell types, are believed to play an important role in the metabolic and physiological molecular pathways of these cells [137,219,221,222]. Nevertheless, increasing concentrations of ROS can cause DNA fragmentation and cell damage [133,137,222], where oxidized bases can lead to DNA

breakages [136,223]. A study by Dietrich et al., 2005, demonstrated that exposure to H_2O_2 significantly increased DNA fragmentation in trout spermatozoa and also reduced the percentage of motile cells. Spermatozoa are more susceptible to oxidative stress than other types of cells because of the polyunsaturated nature of the membrane lipids and the low antioxidant enzymatic activity [133].

Our hypothesis was that extracellular conditions affect *A. zeteki* spermatozoa: 1) percentage motility and Forward Progressive Motility (FPM); 2) duration of motility, 3) Morphology, and 4) DNA integrity, independently of the hormonal stimulation treatment utilized for its collection. The main purpose of the study was to compare the effect of three different extracellular conditions on *A. zeteki* sperm functionality and quality, by assessing the long-term effect of hormonal treatments, time and exposure to different extracellular environments on the percentage motility and FPM of these cells, and therefore determine the best environment for PGF sperm handling, storage and processing for the future applications of ARTs in the species.

Materials and Methods

Animals and Approvals

Thirty adult males *Atelopus zeteki* (>2 year old, average weight of 3.81±0.41 g) were obtained from the Species Survival Plan (SSP) population from the Maryland Zoo in Baltimore, MD. Animal Use Protocols were submitted for the Institutional Animal Care and Use Committee (IACUC) approval from the University of Maryland, the Smithsonian National Zoological Park (SNZP) in Washington, D. C., and Maryland Zoo in Baltimore, MD. After obtaining IACUC approvals, the animals were transferred from

the Maryland Zoo to the SNZP and were placed 30 days under quarantine. One individual died during quarantine and two more died in the next two years.

Six terraria (Exo-Terra Terrarium, 24"x18"x24") were adapted and designed to accommodate five males per tank. Each tank was fitted with live potted plants and live moss as substrate. The recommended humidity in the tanks (60% to 90%) was achieved with the use of an automated misting system that works with reverse osmosis filtered water and that was set up to mist once every hour for two minutes. Excess water was discarded utilizing a flow through drainage system. Temperature ranged from 20 to 22 °C with the use of a window unit air conditioner. All frog terraria were supplied with a UVb Light System, (Zoomed's 10.0 T5 High Output Bulbs) to avoid hypovitaminosis D_3 and help with proper bone growth [141].

Frogs were fed four times per week. Food items included; one-week-old crickets dusted with a calcium carbonate powder containing vitamin D_3, wingless fruit flies and bean beetles. Items were fed on a rotating basis.

For identification purposes, four photographs of each animal were taken to distinguish males utilizing their spot patterns and an alpha-numeric code was assigned to each as follows: PGF01 to PGF30, where PGF stands for Panamanian Golden Frog.

Hormone Stimulation and Sperm Collection

Hormonal stimulations were administered via intracoelomic injection with the help of a Veterinarian from the National Zoo [11,62]. Treatments were carried out once a week for twelve weeks by stimulating groups of three randomly selected males with 40 μl of one of three hormone treatments making a total of twelve individuals by treatment from which data was taken only from twenty five. Within each group one male received one of

the following treatments: **1)** Treatment 1: 4 μg of GnRH-A [des-Gly[10], D-Ala[6], Pro-NHEt[9]] (Sigma-Aldrich Corporation, St. Louis, MO, USA) per gram of bodyweight, 2**)** Treatment 2: 10 IU of hCG [CG5 Chorionic gonadotropin human] (Sigma-Aldrich Corporation, St. Louis, MO, USA) per gram of bodyweight, **3)** Treatment 3: a cocktail of 0.4 μg of GnRH-A [des-Gly[10], D-Ala[6], Pro-NHEt[9]] (Bachem Americas Inc., Torrance, CA, USA) and 10 μg of Metoclopramide hydrochloride (Sigma-Aldrich Corporation, St. Louis, MO, USA) per gram bodyweight (Amphiplex Method) (Trudeau et al., 2010). Dose selection was based on approximates for those that have successfully promoted spawning in other species of amphibians [30,81,119].

After injections, males were placed inside ventilated plastic containers with water soaked paper towel on the bottom and covered from light to reduce stress. Spermic urine was collected by gently inserting a small catheter (0.023" I.D. x 0.039" O.D., Micro Medical Tubing, 85 Durometer Vinyl, Scientific Commodities Inc., Lake Havasu City, AZ, USA) in the cloacae [65].

Two sperm samples per male were collected (C1 and C2), for each collection, samples were separated in three aliquots (conditions) as follows: raw spermic urine kept at room temperature (RT) (21.15±0.05 °C), raw spermic urine kept at 4 °C (4 °C) and 1:1 dilution in water kept at room temperature (WD).

Sperm processing and analysis

Basic metrics were utilized to define the general characteristics of *A. zeteki's* spermic urine. Total volume of excretion was determined with the use of a micropipette. Sperm concentration was assessed with the use of a hemocytometer; 10 μl of spermic urine were added to the chamber and counted following the five square rule. The osmolality and pH

of the spermic urine were obtained with the use of an osmometer (Vapro Osmometer 5520, Wescor, Inc, South Logan, Utah, USA) and pH strips (pH-indicator strips pH 5.0 - 10.0, EMD Millipore, Billerica, MA, USA) respectively.

Evaluation of Sperm Motility and Forward Progressive Motility

Percentage of sperm motility was determined by counting all the cells with flagellar movement from a total of 100 cells under an Olympus BX41 microscope at a 400X magnification. Percentage forward progresive motility (FPM) was obtained by counting all the cells expressing forward motility relative to the 100 cells that were expressing any kind of flagellar movement.

Percentage motility and FPM were determined at three different time points after collection for each of the conditions in all the hormone treatments. Ater determing there was no difference between C1 and C2, data from both collections was averaged and presented as follows: 0:00 minutes (at collection time); 0:23±0:005 minutes after collection and 0:46±0:005 minutes after collection.

Morphology

Morphology was assessed only at 0:23 m after collection, because of limited spermic urine volume available. Samples from all hormone treatment were pooled together since our previous study revealed hormone treatments have no effect on the morphology of A. zeteki spermatozoa. Samples were fixed in 4% paraformaldehyde solution diluted in PBS. Fixed cell were stained with Coomassie Blue (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) and mounted with Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, PA, USA). Sperm morphology was evaluated at a 1000X magnification under an Olympus BX41 microscope in two different

ways: 1) normal and abnormal general classification and 2) specific assessment by morphological trait. For the first assessment, a maximum of 100 cells were assessed for every sample and classified as normal if every morphological parameter of the cell was intact (acrosome, head and tail) and as abnormal if at least one of the parameters was not intact. For the second evaluation, a maximum of 100 cells per sample were evaluated and assessed for acrosomal integrity [normal (intact) and abnormal (not intact, missing)], head [normal (intact) and abnormal (bent, broken)] and tail [normal (intact) and abnormal (coiled, with cytoplasmic droplet or broken)]. Images of the cells were acquired with a SPOT RT3™ Color Slider microscope camera and the SPOT Advanced Software (SPOT Imaging Solutions, Sterling Heights, MI, USA).

DNA Integrity

DNA integrity was assessed only at 0:23 m after collection, because of limited spermic urine volume available. Samples from all hormone treatment were pooled together since our previous study revealed hormone treatments have no effect on the DNA integrity of *A. zeteki* spermatozoa. The assessment was made with the use of the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique (DeadEnd™ Colorimetric TUNEL System, Promega Corporation, Madison, WI, USA), with an in situ cell detection kit, following the manufacturer's instructions. Ejaculated spermatozoa used in this assay were previously fixed with 4% paraformaldehyde and kept at 4°C until analyzed. Slides were prepared by smearing the samples and letting them air-dry. Slides were then gently rinsed twice with PBS for 5 minutes. Cells were permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature. Slides were again gently rinsed twice with PBS for 5 minutes followed by incubation with the

equilibration buffer for 10 minutes at room temperature. Then, 100 μl of previously prepared rTdT reaction mix were added to the slides. After covering with plastic coverslips, slides were incubated at 37°C for 60 minutes in a humidified chamber. The reaction was terminated by removing the coverslips and adding 200 μl of 2X SCC to each slide using a pipette, followed by incubation for 15 minutes at room temperature. Slides were rinsed twice with PBS for 5 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 5 minutes. Slides were again rinsed twice with PBS for 5 minutes followed by incubation with 200 μl of Streptavidin Horseradish Peroxidase for 30 minutes. Slides were rinsed twice with PBS for 5 minutes and developed with 3,3′-diaminobenzidine. Slides were mounted with Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, PA, USA) and analyzed under an Olympus BX41 microscope. One hundred cells were counted per slide and classified as intact or fragmented, those with DNA fragmentation were stained dark brown.

Testicular spermatozoa from eleven euthanized males from another study were used as a control for the TUNEL assay. Testes were removed after dissection, macerated in 1 mL of Amphibian Ringer Solution and fixed with 4% paraformaldehyde. Cells were analyzed following the same procedure described above.

Statistical analysis

All statistics were carried out utilizing the GraphPad Prism 6.05 software (GraphPad Prism 6.05, 2014, GraphPad Software, Inc., La Jolla, CA, USA).

Percentage sperm motility and FPM were compared for each hormone treatment, comparing conditions and times after collection and for each time point, comparing

hormone treatments and conditions using 3 x 3 Two-Way ANOVA followed by a Tukey's test for multiple comparisons.

The effect of extracellular conditions on morphology was assessed using One-Way ANOVA followed by a Tukey's test for multiple comparisons. The Brown-Forsythe test was conducted to determine homogeneity of variances for all the One-Way ANOVA analysis.

DNA fragmentation was compared among conditions using Two-Way ANOVA followed by a Tukey's test for multiple comparisons to determine differences among the treatments.

All statistical analysis were considered significantly different at p<0.05.

Results

Evaluation of Sperm Motility, Forward Progressive Motility and duration of motility

A two-way ANOVA was conducted that examined the effect of extracellular conditions and time after collection on percentage sperm motility of *A. zeteki* in the three hormone treatments: GnRH-A 0.4 µg/µl (Fig. 11), hCG 1 IU/µl (Fig. 12) and Amphiplex (Fig. 13).

Results from the GnRH-A 0.4 µg/µl treatment showed there was no interaction between the effects of extracellular conditions and time after collection ($F_{(4, 64)}$=0.6632, p>0.05). However, there was a significant main effect for the extracellular condition factor ($F_{(2, 64)}$ = 13.79, p<0.0001), and a significant main effect for the time after collection factor ($F_{(2, 64)}$ = 6.823, p = 0.0021). Suggesting that both, extracellular condition and time after collection independently affect the percentage sperm motility.

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A multiple comparison test revealed that percentage sperm motility significantly differed between RT samples at 0:00 m and WD samples at 0:23 m (Mean ± SD 95.28 ± 5.48%; 55.88 ± 28.19%, adjusted $p = 0.0133$) and at 0:46 m (Mean ± SD 95.28 ± 5.48%; 48.75 ± 30.81%, adjusted $p = 0.0016$). Samples at 4 °C (0:00) min also differed from WD samples at 0:23 min (Mean ± SD 93.00 ± 10.84%; 55.88 ± 28.19%, adjusted $p = 0.0241$) and at 0:46 min (Mean ± SD 93.00 ± 10.84%; 48.75 ± 30.81%, adjusted $p = 0.0031$), while 4 °C (0:23) min only varied from WD 0:46 min (Mean ± SD 87.88 ± 13.48%; 48.75 ± 30.81%, adjusted $p = 0.0140$).
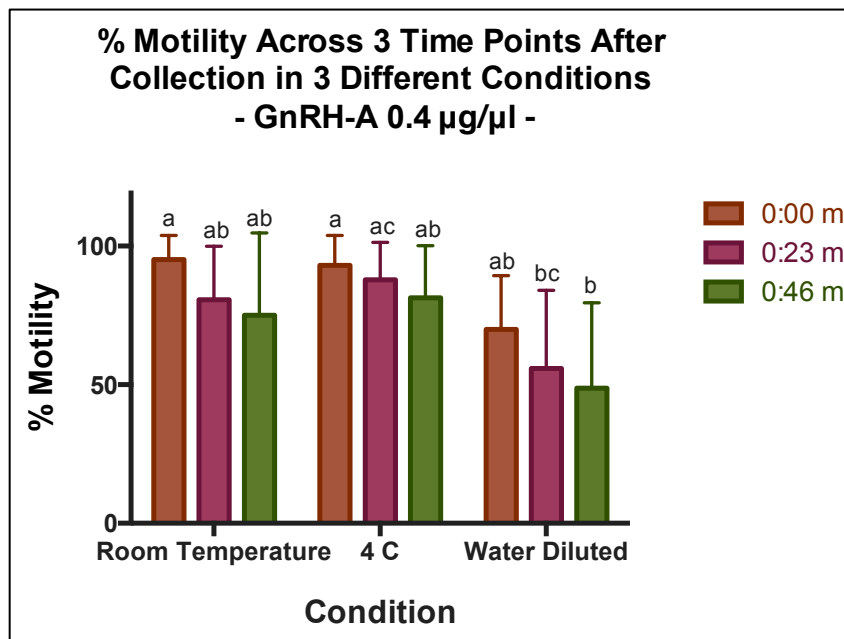
Figure 11. Percentage sperm motility by condition over time - GnRH-A 0.4 μg/μl. Mean ± SD percentage sperm motility, n=8, 2 replicates. Two-way ANOVA main interaction effect ($F_{(4, 64)} = 0.6632$, $p = 0.6198$), main extracellular condition effect ($F_{(2, 64)} = 13.79$, $p<0.0001$) and main time after collection effect ($F_{(2, 64)} = 6.823$, $p = 0.0021$). Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

Results from the hCG 1 IU/μl treatment also showed there was no interaction between the effects of extracellular conditions and time after collection ($F_{(4, 69)} = 0.7347$, $p>0.05$). Conversely, there was a significant main effect for the extracellular condition factor ($F_{(2, 69)} = 14.14$, $p<0.0001$), and a significant main effect for the time after collection factor ($F_{(2, 69)} = 5.819$, $p = 0.0046$).

A multiple comparison test revealed significant differences between RT samples at 0:00 min and WD samples at 0:46 min (Mean ± SD 95.75 ± 5.46%; 50.20 ± 34.13%, adjusted $p<0.0001$), RT at 0:23 min and WD at 0:46 min (Mean ± SD 90.94 ± 8.74%; 50.20 ± 34.13%, adjusted $p = 0.0006$), and RT at 0:46 min and WD at 0:46 min (Mean ± SD 79.80 ± 23.21%; 50.20 ± 34.13%, adjusted $p = 0.0182$). Samples at 4 °C (0:00) min also differed from WD samples at 0:46 min (Mean ± SD 91.83 ± 9.73%; 50.20 ± 34.13%, adjusted $p = 0.0004$), 4 °C at 0:23 min and WD at 0:46 min (Mean ± SD 90.44 ± 11.84%; 50.20 ± 34.13%, adjusted $p = 0.0007$), and 4 °C at 0:46 min varied from WD 0:46 min (Mean ± SD 85.05 ± 15.56%; 50.20 ± 34.13%, adjusted $p = 0.0024$).
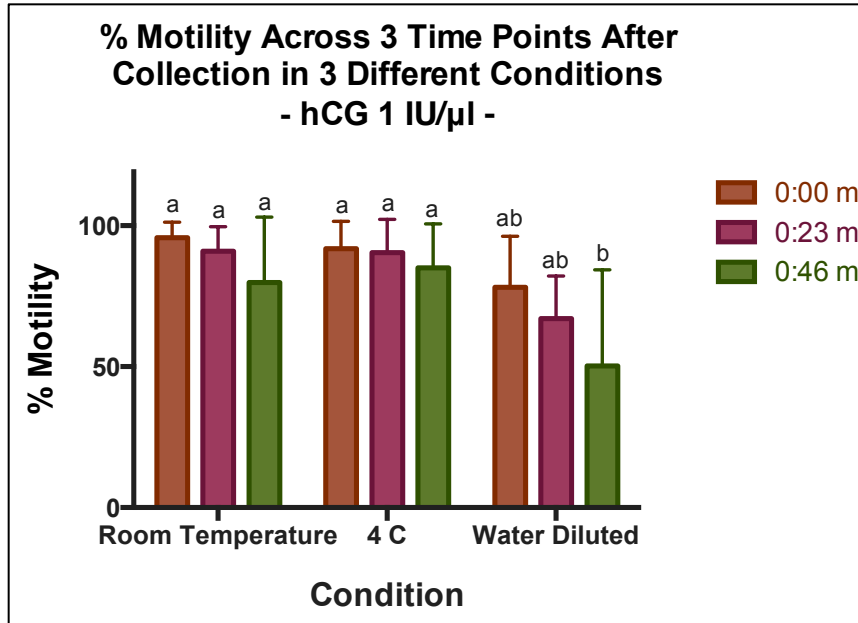
Figure 12. Percentage sperm motility by condition over time - hCG 1 IU/μl. Mean±SD

percentage sperm motility, n=8, 2 replicates. Two-way ANOVA main interaction effect

($F_{(4, 69)}$ = 0.7347, p = 0.5715), main extracellular condition effect ($F_{(2, 69)}$ = 14.14,

$p<0.0001$) and main time after collection effect ($F_{(2, 69)}$ = 5.819, p = 0.0046). Tukey's post

hoc test results are shown as letters above the columns. Means not sharing the same letter

are significantly different.

A main interaction effect between extracellular conditions and time after

collection was observed in the Amphiplex treatment ($F_{(4, 54)}$ = 5.819, p = 0.0125). The

main extracellular condition effect ($F_{(2, 54)}$ = 53.04, $p<0.0001$) and the main time after

collection effect ($F_{(2, 54)}$ = 21.15, $p<0.0001$) were also significant.

A multiple comparison test revealed significant differences between RT at 0:00 m

and WD at 0:23 min (Mean ± SD 89.57 ± 9.71%; 47.14 ± 6.18%, adjusted $p<0.0001$), RT

at 0:00 min and WD at 0:46 min (Mean ± SD 89.57 ± 9.71%; 30.36 ± 16.98%, adjusted p

= 0.0006), RT at 0:23 min and WD at 0:23 min (Mean ± SD 83.00 ± 7.17%; 47.14 ±

6.18%, adjusted p = 0.0001), RT at 0:23 min and WD at 0:46 min (Mean ± SD 83.00 ±

7.17%; 30.36 ± 16.98%, adjusted p<0.0001), RT at 0:46 min and WD at 0:23 min (Mean

± SD 78.36 ± 7.17%; 47.14 ± 6.18%, adjusted p = 0.0008), RT at 0:46 min and WD at

0:46 min (Mean ± SD 78.36 ± 7.17%; 30.36 ± 16.98%, adjusted p<0.0001), 4 °C at 0:00

min and WD at 0:23 min (Mean ± SD 88.93 ± 8.36%; 47.14 ± 6.18%, adjusted

p<0.0001), 4 °C at 0:00 min and WD at 0:46 min (Mean ± SD 88.93 ± 8.36%; 30.36 ±

16.98%, adjusted p<0.0001), 4 °C at 0:23 min and WD at 0:46 min (Mean ± SD 79.43 ±

4.69%; 30.36 ± 16.98%, adjusted p<0.0001), 4 °C at 0:46 min and WD at 0:23 min

(Mean ± SD 72.28 ± 18.35%; 47.14 ± 6.18%, adjusted p=0.0136), 4 °C at 0:46 min and

WD at 0:46 min (Mean ± SD 72.28 ± 18.35%; 30.36 ± 16.98%, adjusted p<0.0001), WD

at 0:00 min and WD at 0:23 min (Mean ± SD 72.36 ± 14.16%; 47.14 ± 6.18%, adjusted

p=0.0132), and WD 0:00 min and WD at 0:46 min (Mean ± SD 72.36 ± 14.16%; 30.36 ±

16.98%, adjusted p<0.0001).
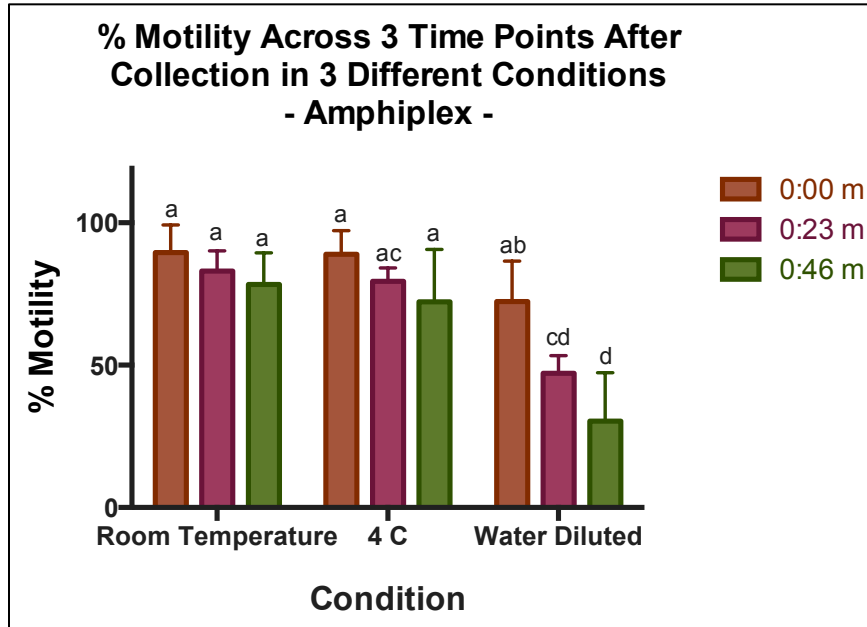
Figure 13. Percentage sperm motility by condition over time - Amphiplex. Mean ± SD percentage sperm motility, n=7, 2 replicates. Two-way ANOVA main interaction effect ($F_{(4, 54)}$ = 3.529, p = 0.0125), main extracellular condition effect ($F_{(2, 54)}$ = 53.04, $p<0.0001$) and main time after collection effect ($F_{(2, 54)}$ = 21.15, $p<0.0001$). Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

Percentage sperm motility was compared among hormone treatments at collection time (0:00) min. There was no interaction effect between extracellular condition and hormone treatment ($F_{(4,60)}$ = 0.4199, $p>0.05$). There was a significant main effect for extracellular condition ($F_{(2,60)}$ = 18.21, $p<0.0001$) and no main effect for hormone treatment ($F_{(2,60)}$ = 0.9279, $p>0.05$).

Specific differences were found between GnRH-A 0.4 μg/μl at RT and WD GnRH-A 0.4 μg/μl (Mean ± SD 96.00 ± 8.54%; 69.94 ± 19.42%, adjusted $p = 0.0027$), GnRH-A 0.4 μg/μl at RT and WD Amphiplex (Mean ± SD 96.00 ± 8.54%; 72.57 ± 14.26%, adjusted $p = 0.0150$), hCG 1 IU/μl at RT and WD GnRH-A 0.4 μg/μl WD (Mean ± SD 95.75 ± 5.46%; 69.94 ± 19.42%, adjusted $p = 0.0031$), hCG 1 IU/μl at RT and WD Amphiplex (Mean ± SD 95.75 ± 5.46%; 72.57 ± 14.26%, adjusted $p = 0.0168$), GnRH-A 0.4 μg/μl at 4 °C and WD GnRH-A 0.4 μg/μl (Mean ± SD 93.00 ± 10.84%; 69.94 ± 19.42%, adjusted $p = 0.0122$), and hCG 1 IU/μl at 4 °C and WD GnRH-A 0.4 μg/μl (Mean ± SD 92.31 ± 8.71%; 69.94 ± 19.42%, adjusted $p = 0.0170$).
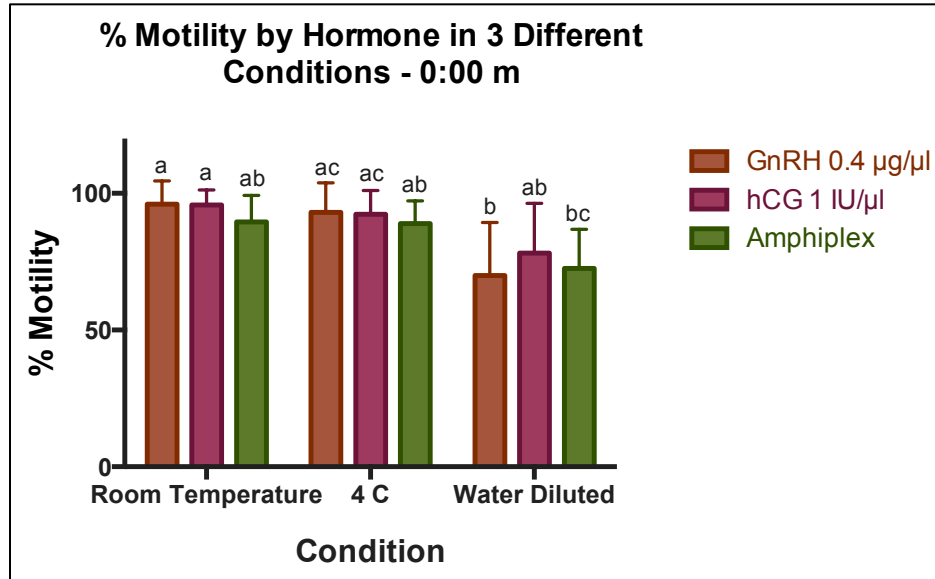
Figure 14. Percentage sperm motility by hormone treatment and condition at 0:00 min after collection, Mean ± SD percentage sperm motility, n=8, 2 replicates. Two-way ANOVA main interaction effect ($F_{(4, 60)} = 0.4199$, $p = 0.7936$), main extracellular condition effect ($F_{(2, 60)} = 18.21$, $p<0.0001$) and main time after collection effect ($F_{(2, 60)} = 0.9279$, $p = 0.4010$). Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

At 0:23 min after collection, there was no main interaction effect between hormone treatments and extracellular condition ($F_{(4, 57)} = 0.2374$, $p>0.05$), no main hormone treatment effect ($F_{(2, 57)} = 2.758$, $p>0.05$), nevertheless, a main extracellular condition affect was detected ($F_{(2, 57)} = 20.99$, $p<0.0001$).

A multiple comparison test revealed significant differences between GnRH-A 0.4 µg/µl at RT and WD GnRH-A 0.4 µg/µl (Mean ± SD 81.25 ± 18.24%; 55.88 ± 28.19%, adjusted $p = 0.0027$), GnRH-A 0.4 µg/µl at RT and WD Amphiplex (Mean ± SD 81.25 ± 18.24%; 47.75 ± 6.90%, adjusted $p = 0.0150$), hCG 1 IU/µl at RT and WD GnRH-A 0.4

μg/μl (Mean ± SD 90.88 ± 14.39%; 55.88 ± 28.19%, adjusted p = 0.0027), hCG 1 IU/μl

at RT and WD Amphiplex (Mean ± SD 90.88 ± 14.39%; 47.75 ± 6.90%, adjusted p =

0.0168), GnRH-A 0.4 μg/μl at 4 °C and WD GnRH-A 0.4 μg/μl (Mean ± SD 87.81 ±

13.54%; 55.88 ± 28.19%, adjusted p = 0.0122), and hCG 1 IU/μl at 4 °C and WD GnRH-

A 0.4 μg/μl (Mean ± SD 90.25 ± 14.27%; 55.88 ± 28.19%, adjusted p = 0.0170).
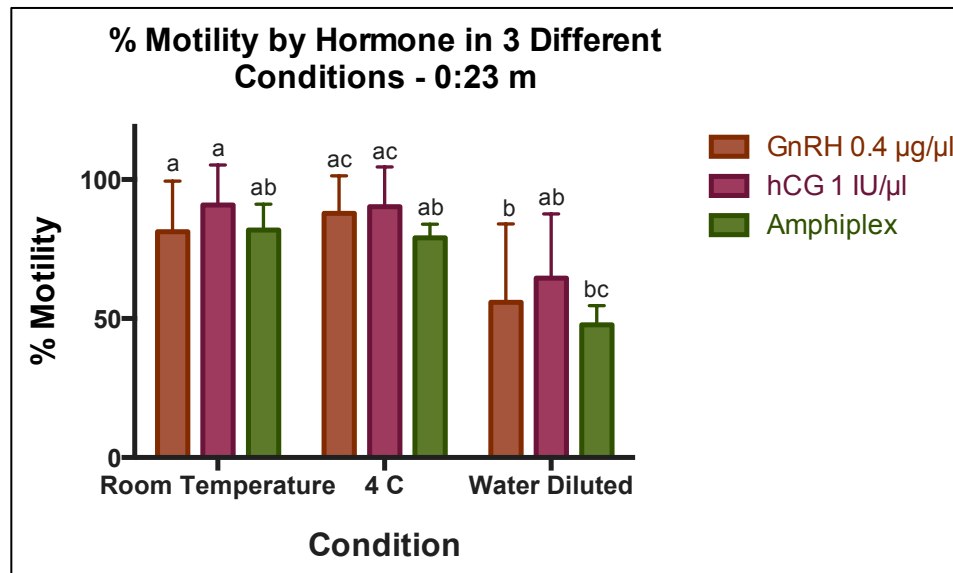
Figure 15. Percentage sperm motility by hormone treatment and condition at 0:23 min

after collection. Mean ± SD percentage sperm motility, n=7, 2 replicates. Two-way

ANOVA main interaction effect ($F_{(4, 57)}$ = 0.2374, p = 0.9161), main extracellular

condition effect ($F_{(2, 57)}$ = 20.99, $p<0.0001$) and main time after collection effect ($F_{(2, 57)}$ =

2.758, p = 0.0719). Tukey's post hoc test results are shown as letters above the columns.

Means not sharing the same letter are significantly different.

At 0:46 min after collection, there was no interaction effect between hormone

treatment and extracellular condition ($F_{(4, 60)}$ = 0.3140, $p>0.05$), no main effect for

hormone treatment ($F_{(2, 60)}$ = 2.375, $p>0.05$) and a significant main effect for extracellular condition ($F_{(2, 60)}$ = 17.48, $p<0.0001$).

Specific differences were found between GnRH-A 0.4 μg/μl at RT and WD Amphiplex (Mean ± SD 74.56±29.19%; 29.86±17.37%, adjusted p=0.0118), hCG 1 IU/μl at RT and WD Amphiplex (Mean ± SD 82.44±29.19%; 29.86±17.37%, adjusted p=0.0015), Amphiplex at RT and WD Amphiplex (Mean ± SD 74.28±16.11%; 29.86±17.37%, adjusted p=0.0179), GnRH-A 0.4 μg/μl at 4 °C and WD Amphiplex (Mean ± SD 81.38±18.77%; 29.86±17.37%, adjusted p=0.0020), hCG 1 IU/μl at 4 °C and WD Amphiplex (Mean ± SD 85.68±16.06%; 29.86±17.37%, adjusted p=0.0006), and Amphiplex at 4 °C and WD Amphiplex (Mean ± SD 70.10±21.10%; 29.86±17.37%, adjusted p=0.0460).
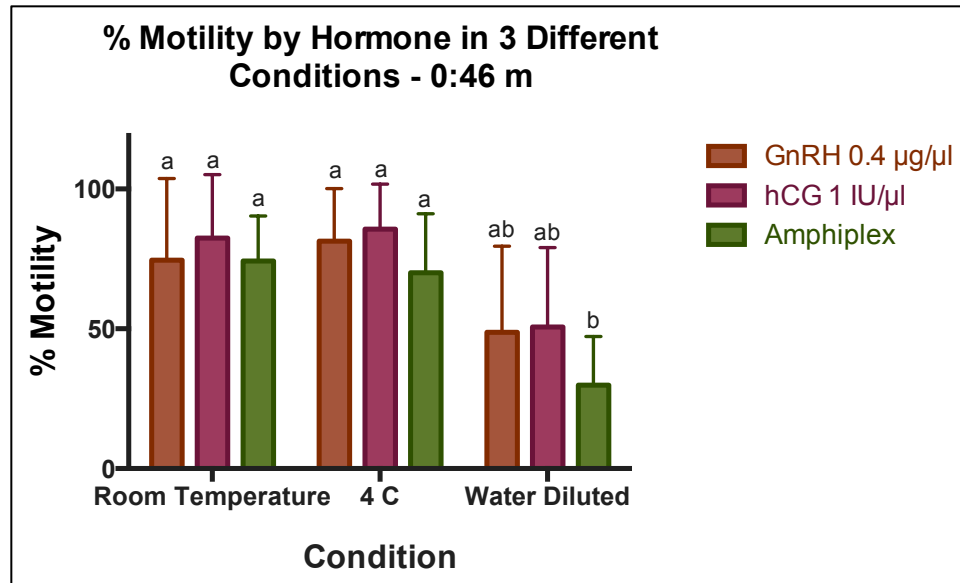
Figure 16. Percentage sperm motility by hormone treatment and condition at 0:46 min after collection. Mean ± SD percentage sperm motility, n=7, 2 replicates. Two-way ANOVA main interaction effect ($F_{(4, 60)}$=0.3140, p=0.8675), main extracellular condition effect ($F_{(2, 60)}$ = 17.48, p<0.0001) and main time after collection effect ($F_{(2, 60)}$=2.375, p=0.1017). Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

A two-way ANOVA was conducted that examined the effect of extracellular conditions and time after collection on percentage forward progressive motility (FPM) of *A. zeteki* in the three hormone treatments: GnRH-A 0.4 µg/µl (Fig. 17), hCG 1 IU/µl (Fig. 18) and Amphiplex (Fig. 19).

Results from the GnRH-A 0.4 µg/µl treatment showed there was no interaction between the effects of extracellular conditions and time after collection ($F_{(4, 63)}$=0.7684, p>0.05). However, there was a significant main effect for the extracellular condition

factor ($F_{(2, 63)}$=122.3, $p<0.0001$), and a significant main effect for the time after collection factor ($F_{(2, 63)}$=12.95, $p<0.0001$).

A multiple comparison test revealed significant differences between RT samples at 0:00 min and WD samples at 0:00 min (Mean ± SD 94.81±5.24%; 43.00±17.91%, adjusted $p<0.0001$), RT samples at 0:00 min and WD samples at 0:23 min (Mean ± SD 94.81±5.24%; 24.75±16.43%, adjusted $p<0.0001$), RT samples at 0:00 min and WD samples at 0:46 min (Mean ± SD 94.81±5.24%; 13.63±15.30%, adjusted $p<0.0001$), RT samples at 0:23 min and WD samples at 0:00 min (Mean ± SD 86.94±8.62%; 43.00±17.91%, adjusted $p<0.0001$), RT samples at 0:23 min and WD samples at 0:23 min (Mean ± SD 86.94±8.62%; 24.75±16.43%, adjusted $p<0.0001$), RT samples at 0:23 min and WD samples at 0:46 min (Mean ± SD 86.94±8.62%; 13.63±15.30%, adjusted $p<0.0001$), RT samples at 0:46 min and WD samples at 0:00 min (Mean ± SD 72.81±23.09%; 43.00±17.91%, adjusted $p=0.0039$), RT samples at 0:46 min and WD samples at 0:23 min (Mean ± SD 72.81±23.09%; 24.75±16.43%, adjusted $p<0.0001$), RT samples at 0:46 min and WD samples at 0:46 min (Mean ± SD 72.81±23.09%; 13.63±15.30%, adjusted $p<0.0001$), 4 °C samples at 0:00 min WD samples at 0:00 min (Mean ± SD 89.94±6.70%; 43.00±17.91%, adjusted $p<0.0001$), 4 °C samples at 0:00 min WD samples at 0:23 min (Mean ± SD 89.94±6.70%; 24.75±16.43%, adjusted $p<0.0001$), 4 °C samples at 0:00 min WD samples at 0:46 min (Mean ± SD 89.94±6.70%; 13.63±15.30%, adjusted $p<0.0001$), 4 °C samples at 0:23 min WD samples at 0:00 min (Mean ± SD 84.81±11.41%; 43.00±17.91%, adjusted $p<0.0001$), 4 °C samples at 0:23 min WD samples at 0:23 min (Mean ± SD 84.81±11.41%; 24.75±16.43%, adjusted

p<0.0001), 4 °C samples at 0:23 min WD samples at 0:46 min (Mean ± SD 84.81±11.41%; 13.63±15.30%, adjusted *p*<0.0001), 4 °C samples at 0:46 min WD samples at 0:00 min (Mean ± SD 76.81±17.14%; 43.00±17.91%, adjusted *p*=0.0006), 4 °C samples at 0:46 min WD samples at 0:46 min (Mean ± SD 76.81±17.14%; 13.63±15.30%, adjusted *p*<0.0001), and WD samples at 0:00 min WD samples at 0:46 min (Mean ± SD 43.00±17.91%; 13.63±15.30%, adjusted *p*=0.0048).

Figure 17. Percentage forward progressive motility by condition over time - GnRH-A 0.4 µg/µl. Mean ± SD percentage sperm motility, n=8, 2 replicates. Two-way ANOVA main interaction effect ($F_{(4, 63)}$=0.7684, *p*=0.5498), main extracellular condition effect ($F_{(2, 63)}$= 122.3, *p*<0.0001) and main time after collection effect ($F_{(2, 63)}$=12.95, *p*<0.0001). Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

Results from the hCG 1 IU/µl treatment showed a main interaction effect between extracellular condition and time after collection ($F_{(4, 63)}$=6.521, p=0.0002), a main extracellular condition effect ($F_{(2, 63)}$=0.50.16, p<0.0001) and a main hormone treatment effect ($F_{(2, 63)}$=31.02, p<0.0001).

Specific differences were found between RT samples at 0:00 m and RT samples at 0:46 min (Mean ± SD 92.68±9.16%; 69.63±12.61%, adjusted p=0.0110), RT samples at 0:00 min and WD samples at 0:23 min (Mean ± SD 92.68±9.16%; 47.19±17.96%, adjusted p<0.0001), RT samples at 0:00 min and WD samples at 0:46 min (Mean ± SD 92.68±9.16%; 31.44±11.88%, adjusted p<0.0001), RT samples at 0:23 min and WD samples at 0:23 min (Mean ± SD 87.06±14.23%; 47.19±17.96%, adjusted p<0.0001), RT samples at 0:23 min and WD samples at 0:46 min (Mean ± SD 87.06±14.23%; 31.44±11.88%, adjusted p<0.0001), RT samples at 0:46 min and 4 °C samples at 0:00 min (Mean ± SD 69.63±12.61%; 91.31±7.39%, adjusted p=0.0214), RT samples at 0:46 min and WD samples at 0:23 min (Mean ± SD 69.63±12.61%; 47.19±17.96%, adjusted p=0.0150), RT samples at 0:46 min and WD samples at 0:46 min (Mean ± SD 69.63±12.61%; 31.44±11.88%, adjusted p<0.0001), 4 °C samples at 0:00 min and WD samples at 0:23 min (Mean ± SD 91.31±7.39%; 47.19±17.96%, adjusted p<0.0001), 4 °C samples at 0:00 min and WD samples at 0:46 min (Mean ± SD 91.31±7.39%; 31.44±11.88%, adjusted p<0.0001), 4 °C samples at 0:23 min and WD samples at 0:23 min (Mean ± SD 83.81±8.53%; 47.19±17.96%, adjusted p<0.0001), 4 °C samples at 0:23 min and WD samples at 0:46 min (Mean ± SD 83.81±8.53%; 31.44±11.88%, adjusted

$p<0.0001$), 4 °C samples at 0:46 min and WD samples at 0:23 min (Mean ± SD 80.94±13.18%; 47.19±17.96%, adjusted $p<0.0001$), and 4 °C samples at 0:46 min and WD samples at 0:46 min (Mean ± SD 80.94±13.18%; 31.44±11.88%, adjusted $p<0.0001$).
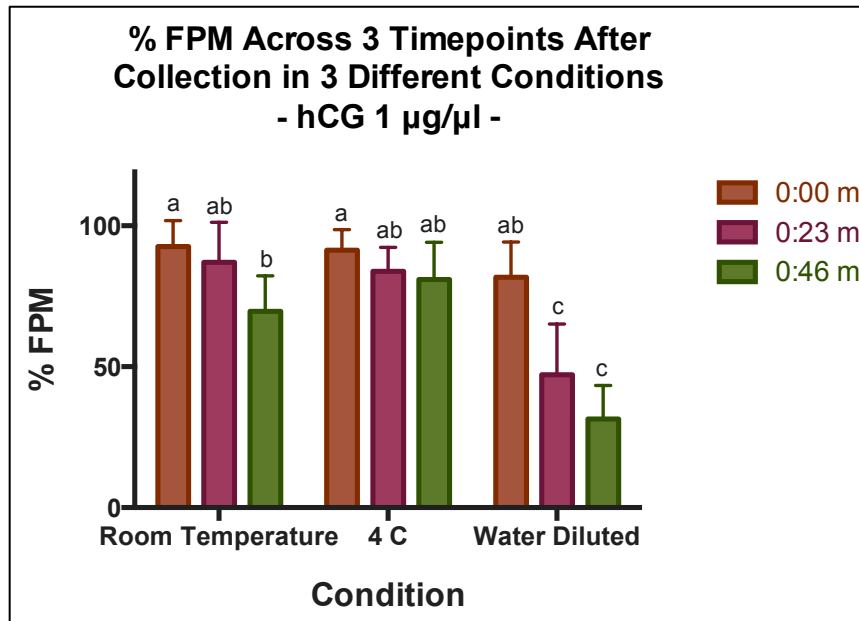
Figure 18. Percentage forward progressive motility by condition over time - hCG 1 IU/μl. Mean ± SD percentage sperm motility, n=8, 2 replicates. Two-way ANOVA main interaction effect ($F_{(4, 63)}$=6.521, p=0.0002), main extracellular condition effect ($F_{(2, 63)}$= 50.16, $p<0.0001$) and main time after collection effect ($F_{(2, 63)}$=31.02, $p<0.0001$). Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

The Amphiplex treatment showed a main interaction effect between extracellular condition and time after collection ($F_{(4, 51)}$=4.557, p=0.0032), a main extracellular

condition effect ($F_{(2, 51)}$=73.15, $p<0.0001$) and a main hormone treatment effect ($F_{(2, 51)}$=31.72, $p<0.0001$).

A multiple comparison test revealed significant differences between RT samples at 0:00 min and 4 °C samples at 0:46 min (Mean ± SD 87.14±13.11%; 61.77±18.69%, adjusted p=0.0167), RT samples at 0:00 min and WD samples at 0:00 min (Mean ± SD 87.14±13.11%; 65.36±13.12%, adjusted p=0.0477), RT samples at 0:00 min and WD samples at 0:23 min (Mean ± SD 87.14±13.11%; 33.64±12.94%, adjusted $p<0.0001$), RT samples at 0:00 min and WD samples at 0:46 min (Mean ± SD 87.14±13.11%; 12.17±8.93%, adjusted $p<0.0001$), RT samples at 0:23 min and 4 °C samples at 0:46 min (Mean ± SD 87.79±9.13%; 61.77±18.69%, adjusted p=0.0128), RT samples at 0:23 min and WD samples at 0:00 min (Mean ± SD 87.79±9.13%; 65.36±13.12%, adjusted p=0.0371), RT samples at 0:23 min and WD samples at 0:23 min (Mean ± SD 87.79±9.13%; 33.64±12.94%, adjusted $p<0.0001$), RT samples at 0:23 min and WD samples at 0:46 min (Mean ± SD 87.79±9.13%; 12.17±8.93%, adjusted $p<0.0001$), RT samples at 0:46 min and WD samples at 0:23 min (Mean ± SD 69.83±12.47%; 33.64±12.94%, adjusted p=0.0001), RT samples at 0:46 min and WD samples at 0:46 min (Mean ± SD 69.83±12.47%; 12.17±8.93%, adjusted $p<0.0001$), 4 °C samples at 0:00 min and 4 °C samples at 0:46 min (Mean ± SD 87.29±8.87%; 61.77±18.69%, adjusted p=0.0158), 4 °C samples at 0:00 min and WD samples at 0:00 min (Mean ± SD 87.29±8.87%; 65.36±13.12%, adjusted p=0.0451), 4 °C samples at 0:00 min and WD samples at 0:23 min (Mean ± SD 87.29±8.87%; 33.64±12.94%, adjusted $p<0.0001$), 4 °C samples at 0:00 min and WD samples at 0:46 min (Mean ± SD 87.29±8.87%;

12.17±8.93%, adjusted $p<0.0001$), 4 °C samples at 0:23 min and WD samples at 0:23 min (Mean ± SD 75.36±13.03%; 33.64±12.94%, adjusted $p<0.0001$), 4 °C samples at 0:23 min and WD samples at 0:46 min (Mean ± SD 75.36±13.03%; 12.17±8.93%, adjusted $p<0.0001$), 4 °C samples at 0:46 min and WD samples at 0:23 min (Mean ± SD 61.77±18.69%; 33.64±12.94%, adjusted $p=0.0052$), 4 °C samples at 0:46 min and WD samples at 0:46 min (Mean ± SD 61.77±18.69%; 12.17±8.93%, adjusted $p<0.0001$), WD samples at 0:00 min and WD samples at 0:23 min (Mean ± SD 65.36±13.12%; 33.64±12.94%, adjusted $p=0.0006$), and WD samples at 0:00 min and WD samples at 0:46 min (Mean ± SD 65.36±13.12%; 12.17±8.93%, adjusted $p<0.0001$).
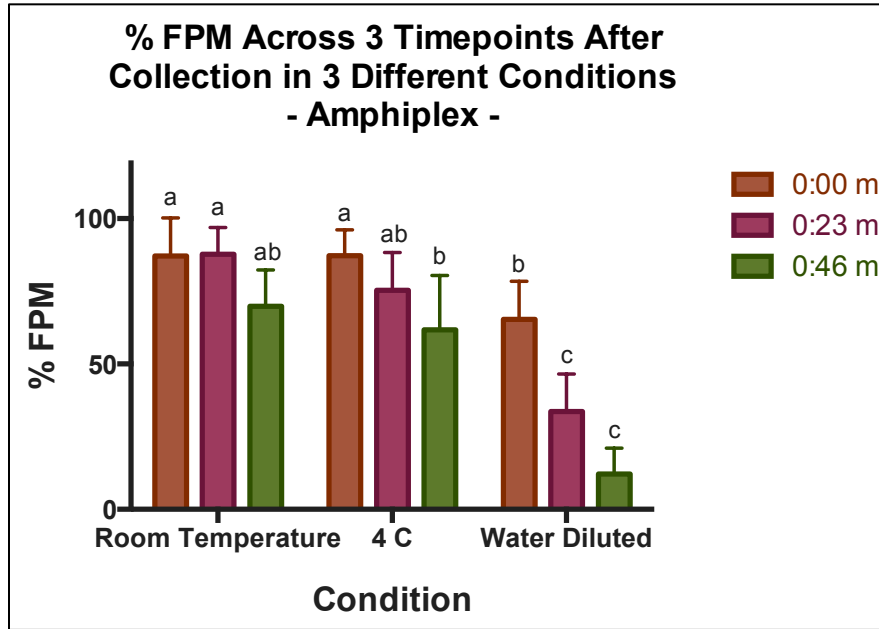
Figure 19. Percentage forward progressive motility by condition over time - Amphiplex. Mean ± SD percentage sperm motility, n=7, 2 replicates. Two-way ANOVA main interaction effect ($F_{(4, 51)}$=4.557, p=0.0032), main extracellular condition effect ($F_{(2, 51)}$= 73.15, $p<0.0001$) and main time after collection effect ($F_{(2, 51)}$=31.72, $p<0.0001$). Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.
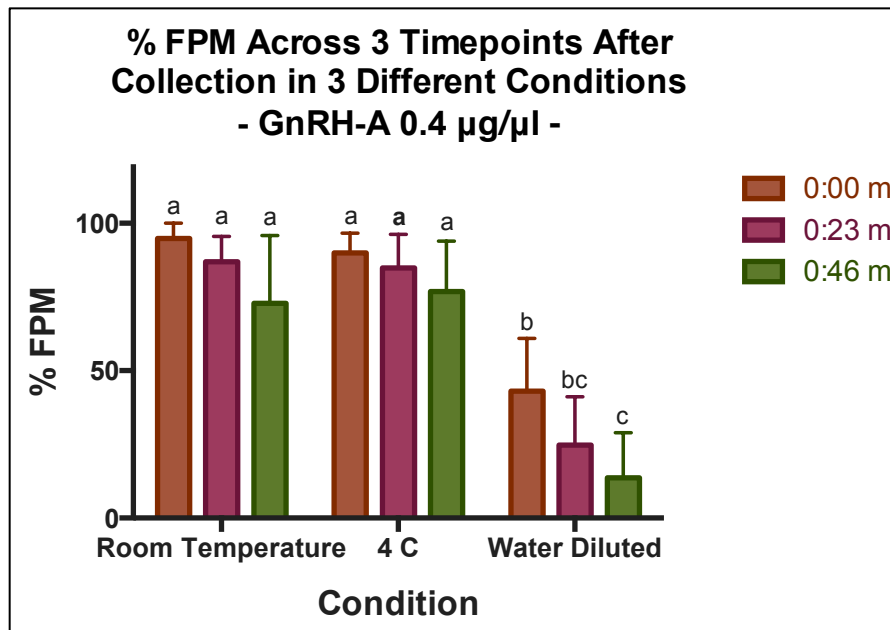
Percentage forward progressive motility was compared among hormone treatments at collection time (0:00 min). There was a significant interaction effect between extracellular condition and hormone treatment ($F_{(4,60)}$=8.353, $p<0.0001$). There also were significant main effects for extracellular condition ($F_{(2,60)}$=36.81, $p<0.0001$) and for hormone treatment ($F_{(2,60)}$=5.908, p=0.0045).

Specific differences were found between GnRH-A 0.4 µg/µl at RT and WD GnRH-A 0.4 µg/µl (Mean ± SD 94.81±5.24%; 43.00±17.91%, adjusted $p<0.0001$), GnRH-A 0.4 µg/µl at RT and WD Amphiplex (Mean ± SD 94.81±5.24%; 73.64±13.32%,

adjusted $p=0.0225$), hCG 1 IU/μl at RT and WD GnRH-A 0.4 μg/μl (Mean ± SD 92.69±9.16%; 43.00±17.91%, adjusted $p<0.0001$), Amphiplex at RT and WD GnRH-A 0.4 μg/μl (Mean ± SD 87.14±13.11%; 43.00±17.91%, adjusted $p<0.0001$), GnRH-A 0.4 μg/μl at 4 °C and WD GnRH-A 0.4 μg/μl (Mean ± SD 90.13±6.63%; 43.00±17.91%, adjusted $p<0.0001$), hCG 1 IU/μl at 4 °C and WD GnRH-A 0.4 μg/μl (Mean ± SD 91.31±7.39%; 43.00±17.91%, adjusted $p<0.0001$), Amphiplex at 4 °C and WD GnRH-A 0.4 μg/μl (Mean ± SD 87.29±8.87%; 43.00±17.91%, adjusted $p<0.0001$), WD GnRH-A 0.4 μg/μl and WD hCG 1 IU/μl (Mean ± SD 43.00±17.91%; 78.48±16.31%, adjusted $p<0.0001$), and WD GnRH-A 0.4 μg/μl and WD Amphiplex (Mean ± SD 43.00±17.91%; 73.64±13.32%, adjusted $p<0.0001$).
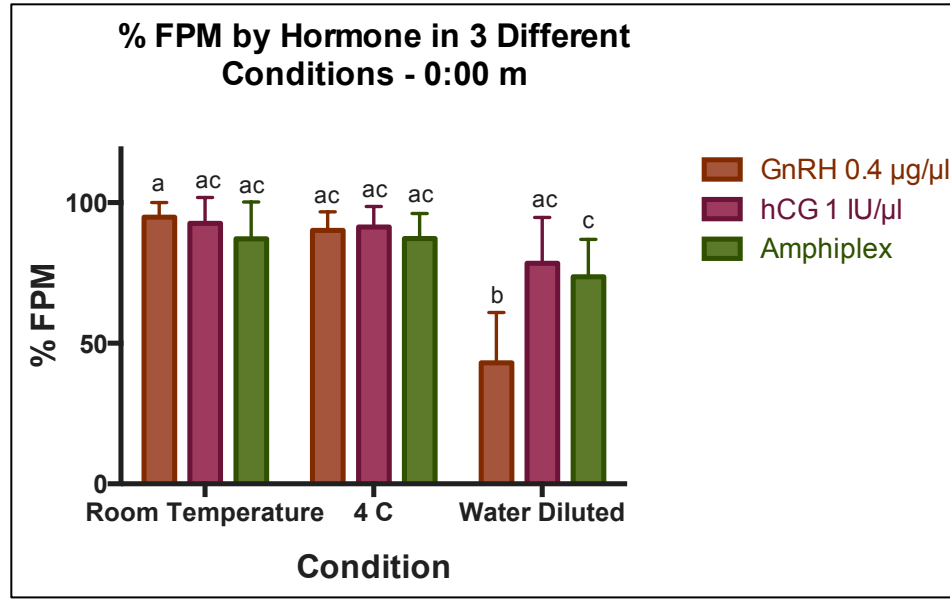
Figure 20. Percentage forward progressive motility by hormone treatment and condition at 0:00 min after collection. Mean ± SD percentage forward progressive motility, n=8, 2 replicates. Two-way ANOVA main interaction effect ($F_{(4, 60)}$=8.353, $p<0.0001$), main extracellular condition effect ($F_{(2, 60)}$ = 36.81, $p<0.0001$) and main hormone treatment effect ($F_{(2, 60)}$=5.908, $p=0.0045$). Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

At 0:23 min after collection, there was no interaction effect between hormone treatment and extracellular condition ($F_{(4, 58)}$=2.399, $p>0.05$), no main effect for hormone treatment ($F_{(2, 58)}$=2.114, $p>0.05$) and a significant main effect for extracellular condition ($F_{(2, 58)}$=102.5, $p<0.0001$).

A multiple comparison test revealed significant differences between GnRH-A 0.4 μg/μl at RT and WD GnRH-A 0.4 μg/μl (Mean ± SD 86.94±8.62%; 24.69±16.33%, adjusted $p<0.0001$), GnRH-A 0.4 μg/μl at RT and WD hCG 1 IU/μl (Mean ± SD 86.94±8.62%; 47.06±18.05%, adjusted $p<0.0001$), GnRH-A 0.4 μg/μl at RT and WD

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Amphiplex (Mean ± SD 86.94±8.62%; 34.42±14.00%, adjusted $p<0.0001$), hCG 1 IU/µl at RT and WD GnRH-A 0.4 µg/µl (Mean ± SD 87.06±14.23%; 24.69±16.33%, adjusted $p<0.0001$), hCG 1 IU/µl at RT and WD hCG 1 IU/µl (Mean ± SD 87.06±14.23%; 47.06±18.05%, adjusted $p<0.0001$), hCG 1 IU/µl at RT and WD Amphiplex (Mean ± SD 87.06±14.23%; 34.42±14.00%, adjusted $p<0.0001$), Amphiplex at RT and WD GnRH-A 0.4 µg/µl (Mean ± SD 88.58±9.73%; 24.69±16.33%, adjusted $p<0.0001$), Amphiplex at RT and WD hCG 1 IU/µl (Mean ± SD 88.58±9.73%; 47.06±18.05%, adjusted $p<0.0001$), Amphiplex at RT and WD Amphiplex (Mean ± SD 88.58±9.73%; 34.42±14.00%, adjusted $p<0.0001$), GnRH-A 0.4 µg/µl at 4 °C and WD GnRH-A 0.4 µg/µl (Mean ± SD 84.81±11.42%; 24.69±16.33%, adjusted $p<0.0001$), GnRH-A 0.4 µg/µl at 4 °C and WD hCG 1 IU/µl (Mean ± SD 84.81±11.42%; 47.06±18.05%, adjusted $p<0.0001$), GnRH-A 0.4 µg/µl at 4 °C and WD Amphiplex (Mean ± SD 84.81±11.42%; 34.42±14.00%, adjusted $p<0.0001$), hCG 1 IU/µl at 4 °C and WD GnRH-A 0.4 µg/µl (Mean ± SD 87.06±14.23%; 24.69±16.33%, adjusted $p<0.0001$), hCG 1 IU/µl at 4 °C and WD hCG 1 IU/µl (Mean ± SD 87.06±14.23%; 47.06±18.05%, adjusted $p<0.0001$), hCG 1 IU/µl at 4 °C and WD Amphiplex (Mean ± SD 87.06±14.23%; 34.42±14.00%, adjusted $p<0.0001$), Amphiplex at 4 °C and WD GnRH-A 0.4 µg/µl (Mean ± SD 75.57±13.09%; 24.69±16.33%, adjusted $p<0.0001$), Amphiplex at 4 °C and WD hCG 1 IU/µl (Mean ± SD 75.57±13.09%; 47.06±18.05%, adjusted $p=0.0028$), Amphiplex at 4 °C and WD Amphiplex (Mean ± SD 75.57±13.09%; 34.42±14.00%, adjusted $p<0.0001$), and WD GnRH-A 0.4 µg/µl and WD hCG 1 IU/µl (Mean ± SD 24.69±16.33%; 47.06±18.05%, adjusted $p=0.0302$).
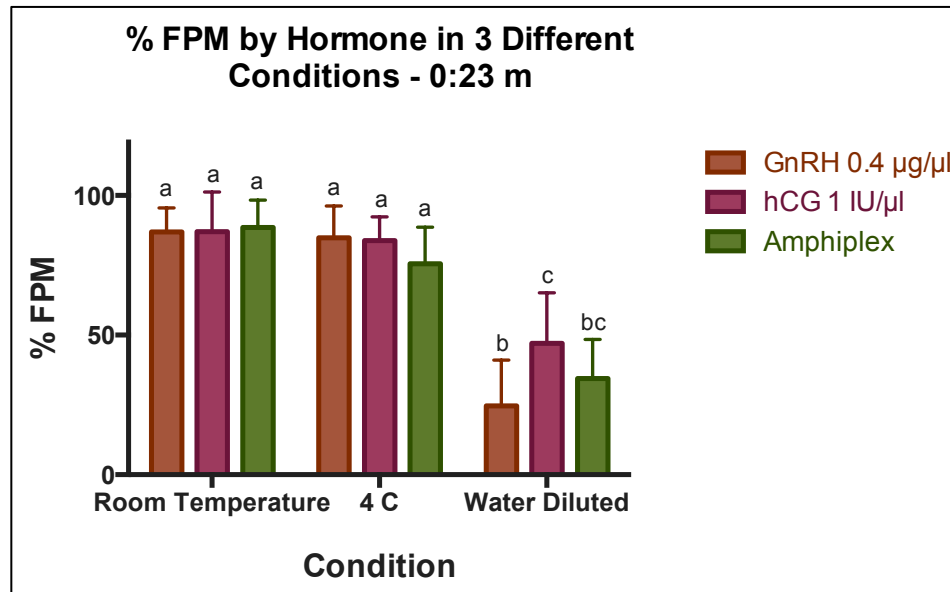
Figure 21. Percentage forward progressive motility by hormone treatment and condition at 0:23 min after collection. Mean ± SD percentage forward progressive motility, n=8, 2 replicates. Two-way ANOVA main interaction effect ($F_{(4, 58)}$=2.399, p=0.0603), main extracellular condition effect ($F_{(2, 58)}$=102.5, p<0.0001) and main hormone treatment effect ($F_{(2, 58)}$=2.114, p=0.1299). Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

At 0:46 min after collection, there was no interaction effect between hormone treatment and extracellular condition ($F_{(4, 55)}$=1.680, p>0.05), no main effect for hormone treatment ($F_{(2, 55)}$=3.138, p>0.05) and a significant main effect for extracellular condition ($F_{(2, 55)}$=79.55, p<0.0001).

Specific differences were found between GnRH-A 0.4 μg/μl at RT and WD GnRH-A 0.4 μg/μl (Mean ± SD 72.21±24.90%; 13.63±15.30%, adjusted p<0.0001), GnRH-A 0.4 μg/μl at RT and WD hCG 1 IU/μl (Mean ± SD 72.21±24.90%;

31.44±11.88%, adjusted p=0.0002), GnRH-A 0.4 μg/μl at RT and WD Amphiplex (Mean ± SD 72.21±24.90%; 12.00±8.83%, adjusted p<0.0001), hCG 1 IU/μl at RT and WD GnRH-A 0.4 μg/μl (Mean ± SD 67.75±13.22%; 13.63±15.30%, adjusted p<0.0001), hCG 1 IU/μl at RT and WD hCG 1 IU/μl (Mean ± SD 67.75±13.22%; 31.44±11.88%, adjusted p=0.0008), hCG 1 IU/μl at RT and WD Amphiplex (Mean ± SD 67.75±13.22%; 12.00±8.83%, adjusted p<0.0001), Amphiplex at RT and WD GnRH-A 0.4 μg/μl (Mean ± SD 70.08±12.60%; 13.63±15.30%, adjusted p<0.0001), Amphiplex at RT and WD hCG 1 IU/μl (Mean ± SD 70.08±12.60%; 31.44±11.88%, adjusted p=0.0010), Amphiplex at RT and WD Amphiplex (Mean ± SD 70.08±12.60%; 12.00±8.83%, adjusted p<0.0001), GnRH-A 0.4 μg/μl at 4 °C and WD GnRH-A 0.4 μg/μl (Mean ± SD 76.81±17.14%; 13.63±15.30%, adjusted p<0.0001), GnRH-A 0.4 μg/μl at 4 °C and WD hCG 1 IU/μl (Mean ± SD 76.81±17.14%; 31.44±11.88%, adjusted p<0.0001), GnRH-A 0.4 μg/μl at 4 °C and WD Amphiplex (Mean ± SD 76.81±17.14%; 12.00±8.83%, adjusted p<0.0001), hCG 1 IU/μl at 4 °C and WD GnRH-A 0.4 μg/μl (Mean ± SD 82.07±13.80%; 13.63±15.30%, adjusted p<0.0001), hCG 1 IU/μl at 4 °C and WD hCG 1 IU/μl (Mean ± SD 82.07±13.80%; 31.44±11.88%, adjusted p<0.0001), hCG 1 IU/μl at 4 °C and WD Amphiplex (Mean ± SD 82.07±13.80%; 12.00±8.83%, adjusted p<0.0001), Amphiplex at 4 °C and WD GnRH-A 0.4 μg/μl (Mean ± SD 61.88±18.80%; 13.63±15.30%, adjusted p<0.0001), Amphiplex at 4 °C and WD hCG 1 IU/μl (Mean ± SD 61.88±18.80%; 31.44±11.88%, adjusted p=0.0199), and Amphiplex at 4 °C and WD Amphiplex (Mean ± SD 61.88±18.80%; 12.00±8.83%, adjusted p<0.0001).
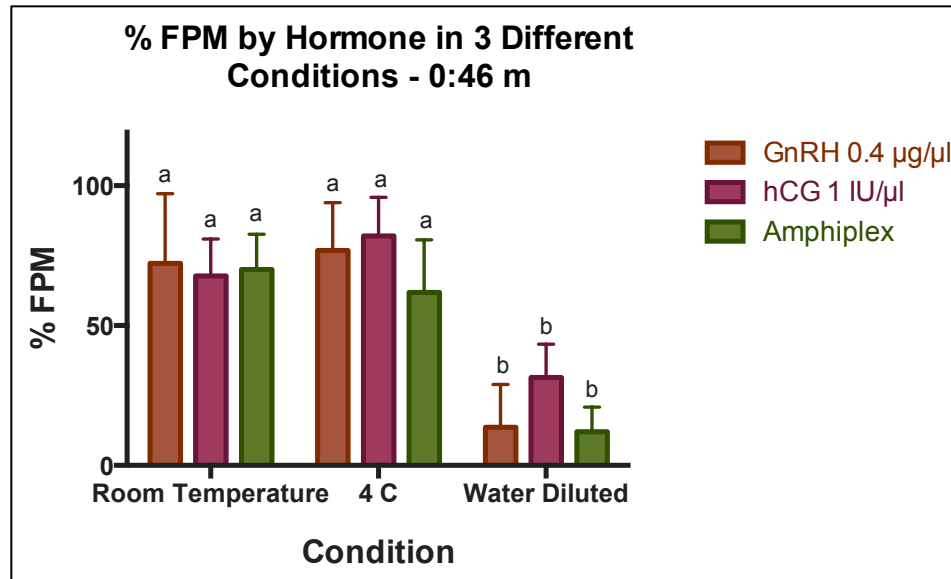
Figure 22. Percentage forward progressive motility by hormone treatment and condition at 0:46 min after collection. Mean ± SD percentage forward progressive motility, n=8, 2 replicates. Two-way ANOVA main interaction effect ($F_{(4, 55)}$=1.680, p=0.1677), main extracellular condition effect ($F_{(2, 55)}$=79.55, p<0.0001) and main hormone treatment effect ($F_{(2, 55)}$=3.138, p=0.0512). Tukey's post hoc test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

Morphology

A one-way ANOVA was conducted that examined the effect of extracellular conditions on the morphology of *A. zeteki* spermatozoa. Results on the general morphology assessment showed no differences in the percentage of normal cells among extracellular conditions ($F_{(2, 37)}$=1.126, p>0.05) with 36.6%, 41.4% and 30.4% percentage of normal cells in the room temperature, 4°C and water diluted condition, respectively. Similarly, no differences among specific morphological traits [Acrosome integrity ($F_{(2, 39)}$=2.656, p>0.05), Head integrity ($F_{(2, 39)}$=1.831, p>0.05) and Tail integrity ($F_{(2, 39)}$=2.961, p>0.05)] were found.

An assessment of the percentage intact DNA revealed significant differences among extracellular conditions ($F_{(2, 17)}$=7.212, p=0.0054). A multiple comparison test showed specific differences between percentage intact DNA in RT and WD samples (Mean ± SD 63.25±15.11%; 32.83±21.20%, adjusted p=0.0152) and between at 4 °C and WD samples (Mean ± SD 77.99±4.57%; 32.83±21.20%, adjusted p=0.0054).
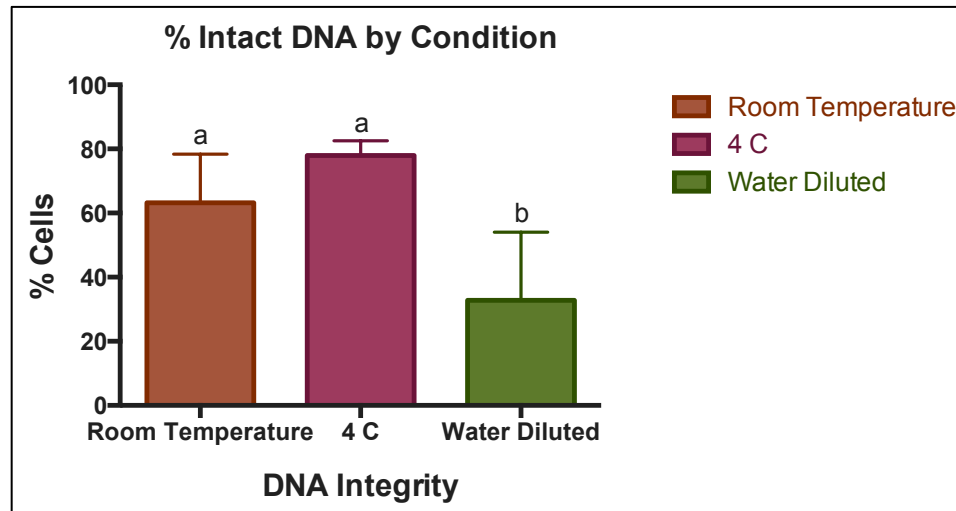
Figure 23. Comparison of percentage intact DNA by extracellular condition. Mean ± SD percentage of cells, n=14. ANOVA ($F_{(6, 17)}$=7.212, p=0.0054). Tukey's multiple comparison test results are shown as letters above the columns. Means not sharing the same letter are significantly different.

Discussion

This study evaluated the effect of different extracellular conditions on the percentage motility and Forward Progressive Motility (FPM), duration of motility, morphology and DNA integrity of hormonally stimulated *A. zeteki* spermatozoa.

__Evaluation of sperm motility, Forward Progressive Motility and duration of__
__motility under three extracellular conditions and hormone treatments__

In our previous study we showed that hormonal stimulation with GnRH-A, hCG and Amphiplex do not negatively affect *A. zeteki* sperm motility at collection time. Nevertheless, we wanted to assess the long-term effect of hormonal treatments, time and exposure to different extracellular environments on the percentage motility and FPM of these cells.

Treatment with GnRH analogs has been widely used to stimulate spawning responses in several species of mammals, fish and amphibians [116,130,224]. Even though stimulation with this hormone has proven effective at increasing the production of sperm (concentration) and improving ejaculate quality parameters in some species [81,98,116,120,164,225,226], the effect of the treatment on amphibian sperm percentage motility, FPM and duration of motility under different extracellular conditions has not been previously investigated.

Spermatozoa obtained after stimulation with GnRH-A 0.4 μg/μl showed that both, extracellular conditions and time after collection affect *A. zeteki* sperm percentage motility and FPM, while no interaction the main effects was observed. Spermatozoa kept in spermic urine at room temperature and at 4 °C showed higher percentage motility sustained for at least 46 min after collection than the water diluted samples. Samples kept in spermic urine at room temperature exhibited high percentage motility at collection time (95.3%) and at 23 min (86%), while 63% of the cells remained motile 46 min after collection. Samples kept in spermic urine at 4 °C also exhibited high percentage motility at collection time (93%) and at 23 min (87.8%), with 81% of the cells still showing motility 46 min after collection. Following dilution in water, percentage motility

decreased from 25% and to 31% at collection time and at 23 min, respectively, compared to the samples kept in spermic urine at room temperature and at 4 °C. Percentage motility dropped to 48.8% in water diluted samples 46 min after collection.

Forward progressive motility of samples obtained after GnRH-A 0.4 μg/μl stimulation were higher in spermic urine samples kept at room temperature and at 4 °C. Spermatozoa kept in spermic urine at room temperature showed high FPM at collection time (94.8%) than at 23 min (86.9%) and 46 min (72.8%) after collection. Samples kept in spermic urine at 4 °C showed a trend of higher FPM sustained over a longer period of time compared to the room temperature condition, although not significant, with 89.9%, 84.8% and 76.8% at collection time, 23 min and 46 min after collection, respectively. In water diluted samples, FPM dramatically dropped, with 43% FPM at collection time, 24.7% at 23 min and 13.6% at 46 min after collection.

The effect of GnRH-A treatment on sperm percentage motility, FPM and duration of motility has been assessed in some species of fish and amphibians, mainly when the cells have been exposed to diluted media. Studies in yellowtail flounder spermatozoa indicate that the percentage of motile cells and their swimming time increase with GnRH-A stimulation after dilution in seawater compared to spermatozoa from non-stimulated fish [116]. Caspian brown trout spermatozoa obtained after treatment with GnRH-A shows increased motility duration after dilution in saline solution [164]. Spermatozoa from *L. laevis* collected after GnRH-A stimulation and diluted in tap water remains motile for at least 80 min after collection at 28 °C [119], while spermatozoa from *Bufo americanus* obtained with the same treatment remains motile for 30 min after collection [81]. Even though these studies do not compare the effect of the treatment in different extracellular

conditions but only in diluted media, they suggest a positive effect of GnRH-A stimulation on these parameters after milt dilution and sperm activation. Our results suggest that *A. zeteki* spermatozoa obtained after stimulation with GnRH-A 0.4 μg/μl could maintain higher percentage motility and FPM for a longer period of time when kept in spermic urine at 4 °C, even though there was no significant difference between this treatment and the samples kept at room temperature, and that exposure to dilution in water negatively affects percentage motility, FPM and duration of motility in this cells.

Comparably to what we observed with GnRH-A treatment, samples obtained after stimulation with hCG 1 IU/μl showed that both, extracellular conditions and time after collection affect *A. zeteki* sperm motility, with no interaction between the main effects. As seen in the samples obtained with GnRH-A 0.4 μg/μl stimulation, spermatozoa obtained after treatment with hCG 1 IU/μl showed higher percentage motility sustained for >46 min in samples kept in spermic urine at room temperature and 4 °C. Samples kept in spermic urine at room temperature exhibited high percentage motility at collection time (95.8%) and at 23 min (90.9%), while 79.8% of the cells remained motile 46 min after collection. Samples kept in spermic urine at 4 °C also exhibited high percentage motility at collection time (91.8%) and at 23 min (90.4%), with 85% of the cells still showing motility 46 min after collection. Following water dilutions, samples showed a marked decrease in percentage motility compared to the two other extracellular conditions. Water diluted samples kept at room temperature, showed 78%, 64.5% and 50.6% motility at collection time, 23 min and 46 min after collection, respectively.

Contrary to what we observed in the sperm percentage motility, FPM under hCG 1 IU/μl stimulation showed a significant interaction between extracellular condition and

time after collection, indicating that the effect of the extracellular condition on FPM depended on the time the samples were exposed to that particular condition. FPM was significantly lower in samples diluted in water while samples kept at room temperature and at 4 °C showed higher FPM with no significant difference between the two conditions. Spermatozoa kept in spermic urine at room temperature showed higher FPM at collection time (92.7%) than at 23 min (87%) and 46 min (69.6%) after collection. Samples kept in spermic urine at 4 °C showed a trend of higher FPM sustained over time as in the previous treatment, although not significant, compared to the room temperature condition, with 91.3%, 83.8% and 80.9% at collection time, 23 min and 46 min after collection, respectively. In water diluted samples, FPM was initially high at collection time (81.7%) and dramatically dropped over the next two time points, with 47.2% at 23 min and 31.4% at 46 min after collection.

Human chorionic gonadotropin has been widely used in the stimulation of spermiation in several species of fish and amphibians with a variety of species-specific responses and different effects on sperm quality parameters [52,57,120,122,127,145,210,227,228]. In the Japanese eel, sperm motility varies over time after hCG stimulation [170], while in the European eel, percentage and duration of motility is lower, compared to pituitary gland extracts and GnRH-A stimulation [151,210]. Treatment with hCG produces lower sperm concentrations with lower percentage motility in *Dendrobates auratus* compared to other species of amphibians [122]. Mansour et al., 2010, found no difference in motility parameters from hCG stimulated vs. non stimulated testicular sperm from *Rana temporaria*. However, the same treatment increases sperm production and motility in *Bufo fowleri* spermatozoa [11]. Still, none of these studies assesses the effect of hCG

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stimulation on sperm motility or FPM over time nor indicate the possible effect of the extracellular environment.

Similarly to the results obtained after GnRH stimulation, these set of results also suggest that spermatozoa obtained after stimulation with hCG 1 IU/μl show higher percentages of motility and FPM maintained for longer periods of time when kept in spermic urine at 4 °C and that dilution in water drastically reduces *A. zeteki* sperm motility and FPM after 46 min of exposure to this condition. Intriguingly, water diluted samples under hCG 1 IU/μl treatment showed higher FPM than water diluted samples obtained with GnRH-A 0.4 μg/μl stimulation.

Stimulation with Amphiplex showed a significant interaction between extracellular condition and time after collection, indicating that, under this treatment the effect of the extracellular condition on the sperm percentage motility depended on the time the samples were exposed to that particular condition. Spermatozoa obtained after treatment with Amphiplex showed higher percentage motility for at least 46 min in samples kept in spermic urine at room temperature and 4 °C. Samples kept in spermic urine at room temperature exhibited high percentage motility at collection time (89.6%) and at 23 min (83%), while 78.4% of the cells remained motile 46 min after collection. Samples kept in spermic urine at 4 °C also exhibited high percentage motility at collection time (88.9%) and at 23 min (79.4%), with 72.3% of the cells still showing motility 46 min after collection. Water diluted samples showed lower percentage motility compared to the two other extracellular conditions. Water diluted samples kept at room temperature, showed 72.6%, 47.8% and 29.9% motility at collection time, 23 min and 46 min after collection, respectively. Similarly to the two previous hormonal treatments,

samples obtained with Amphiplex stimulation also exhibited higher percentage motility and longer duration of motility in samples kept in spermic urine at room temperature and at 4 °C. Dilution in water also decreased these metrics drastically in this treatment group.

As observed in percentage motility, when assessing FPM, Amphiplex stimulation showed a significant interaction between extracellular condition and time after collection, indicating that, under this treatment, the effect of the extracellular condition depended on the time the samples were exposed to that particular condition. In this treatment, FPM was lower in water-diluted samples than in the rest of the extracellular conditions. Samples kept at room temperature and at 4 °C showed higher FPM, while there was no significant difference between the two conditions. Spermatozoa kept in spermic urine at room temperature showed high FPM at collection time (87.1%), at 23 min (87.7%) and lower FPM at 46 min (69.8%) after collection. Samples kept in spermic urine at 4 °C also showed high FPM sustained over time, with 87.3%, 75.4% and 61.7% at collection time, 23 min and 46 min after collection, respectively. In water diluted samples, FPM was higher at collection time (65.3%) and dramatically dropped over the next two time points as seen in the hCG 1 IU/μl treatment, with 33.6% at 23 min and 12.2% at 46 min after collection.

Amphiplex is a hormone cocktail that contains 0.4 μg of GnRH-A [des-Gly^{10}, D-Ala^6, Pro-NHEt^9] and 10 μg of Metoclopramide hydrochloride, a dopamine antagonist, that has been successfully used in the stimulation of spawning in amphibians [30,148]. Other combinations of GnRH-A (mammalian or salmonid) and dopamine antagonists, like pimozide and domperidone, have been used for induction of ovulation and spermiation in fish and amphibians as well [117,159]. A study on *Barbus barbus* spermatozoa stimulated

with two preparations of GnRH-A with either metoclopramide (Ovopel®) or domperidone (Ovaprim®) showed that sperm percentage motility did not change between the treatment groups and the controls (NaCl), however, treatment with Ovopel increased FPM, although not significantly [159]. Similarly, these two preparations, in addition to carp pituitary extracts, were used for the stimulation of Crucian carp with no difference on sperm percentage motility nor FPM among the groups [211]. Common carp spermatozoa obtained after treatment with Ovaprim® and diluted in water shows a FPM of about 30 sec after activation [73]. Nevertheless, Ovopel® treatment in the European smelt produced spermatozoa with higher percentage motility compared to the controls [212]. None of these studies evaluated the long-term effect of these hormonal preparations or the effect of extracellular conditions on the sperm percentage motility, FPM and duration of motility. Treatments with these kinds of hormone cocktails are very efficient at increasing sperm concentration, while the effects on sperm motility are very variable among species.

Several studies point out the possibility of a wider effect of these hormone preparations involving dopamine antagonists on the steroid composition of the seminal plasma and a possible influence on sperm quality parameters [159,212]. In our study, percentage motility, FPM and duration of motility were slightly lower in the Amphiplex treatment compared to the GnRH-A and hCG treatments. It remains to be investigated the effect of Amphiplex or metoclopramide alone on the spermic urine composition and its relationship with *A. zeteki* sperm motility.

When comparing the sperm percentage motility between hormone treatments and extracellular conditions at 3 different time points after collection, a main effect of extracellular condition was detected for all time points, with no significant interaction

between the two factors, indicating that *A. zeteki* sperm motility is not affected by hormone treatments but by the extracellular condition they are exposed to.

At collection time (0:00 min), samples kept in spermic urine at room temperature and at 4 °C showed no significant difference in percentage motility, while in water diluted samples, those obtained with GnRH-A 0.4 μg/μl treatment, showed lower percentage motility (69.9%) at collection time than those obtained with hCG 1 IU/μl (78.2%) and Amphiplex (72.6%), with hCG 1 IU/μl samples showing a slight increase, although not significant, in percentage motility compared to the other hormone treatments.

At 23 min after collection, samples kept in spermic urine at room temperature and at 4 °C showed no significant difference in percentage motility as observed at collection time while a significant difference was detected between these and water diluted samples. Among water diluted samples, although no significant, those obtained with Amphiplex showed lower percentage motility (47.8%) at 23 min after collection than those obtained with GnRH-A 0.4 μg/μl (55.9%) and hCG 1 IU/μl (64.5%) treatments, with hCG 1 IU/μl samples again showing a slight increase, although not significant, in percentage motility compared to the other hormone treatments in the water diluted extracellular condition.

Similarly to the two previous time points, at 46 min after collection, samples kept in spermic urine at room temperature and at 4 °C showed no significant difference in percentage motility. Water diluted samples obtained with Amphiplex showed the lowest percentage motility (29.9%), than those obtained with GnRH-A 0.4 μg/μl (48.8%) and hCG 1 IU/μl (50.6%) treatments, with hCG 1 IU/μl samples again showing a slight

increase, although not significant, in percentage motility compared to the other hormone treatments in the water diluted extracellular condition.

When comparing the sperm FPM between hormone treatments and extracellular conditions at three different time points after collection, an interaction between the main effects was observed at collection time but absent at 23 mi and 46 min after collection, where just the main effect of extracellular condition was significant. At collection time, this interaction indicates that the differences in *A. zeteki* sperm FPM observed under a specific extracellular condition depend on the hormonal treatment that was used for stimulation, while in the next two time points FPM is not affected by the hormone treatments but by the extracellular condition they are exposed to.

At collection time (0:00 min), samples obtained with the three hormonal treatments kept in spermic urine at room temperature, at 4 °C and water diluted samples collected after stimulation with hCG 1 IU/µl and Amphiplex showed no significant difference in FPM. All samples kept in spermic urine at room temperature and at 4 °C showed high FPM with a minimum of 87.2%, while water diluted samples under all hormonal treatments showed decreased FPM compared to the other two conditions with only samples collected after GnRH-A 0.4 µg/µl stimulation showing a significant decrease in FPM (43%). At his time point, as observed in sperm percentage motility, water diluted samples obtained after stimulation with hCG 1 IU/µl showed a not significant, but higher FPM that most of the hormone treatments.

At 23 min after collection, sperm collected after stimulation with the three treatments showed similar high FPM percentage at room temperature and at 4 °C, with a minimum of 75.6%, and a not significant but higher FPM observed at room temperature

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at this time point. On the other hand, water diluted samples showed a significant decrease in FPM compared to the other two conditions. hCG 1 IU/μl stimulated water diluted samples showed a significant higher FPM that water diluted samples obtained after treatment with GnRH-A 0.4 μg/μl, and a higher, but not significant FPM than the Amphiplex treatment.

Similar to what we observed at the previous time point, at 46 min after collection, samples obtained with the three hormone treatments showed no significant difference in FPM between the room temperature and 4 °C conditions, nevertheless, a trend of higher FPM was observed in samples kept at 4 °C. Water diluted samples showed a significant decrease in FPM compared to the other two conditions, and as reported at 23 min after collection, a not significant but higher FPM was observed in water diluted samples obtained after hCG 1 IU/μl stimulation compared to water diluted samples obtained with the other two hormone treatments.

Interestingly, treatment with hCG 1 IU/μl showed a trend of increased percentage motility and FPM in most of the extracellular conditions and time points. These trends of increasing motility parameters, although no significant, might indicate a positive effect of stimulation with hCG 1 IU/μl in the percentage motility, FPM and duration of motility of *A. zeteki* spermatozoa. Instead, stimulation with Amphiplex, while in our previous study proved to be the best treatment for the collection of higher sperm concentrations, seemed to reduce the duration of sperm motility at 23 and 46 min after collection when spermatozoa were exposed to media of lower osmolalities, while it maintains good enough motility parameters at room temperature and at 4 °C, as also seen with GnRH-A 0.4 μg/μl stimulation.

The interaction that we observed between hormonal treatments and extracellular conditions when assessing FPM at collection time, might be related to the following issues: 1) forward progression requires more energy from the cell storages and extracellular media than steady flagellar twitching or beating, 2) hormones could be changing the composition of spermic urine and this effect might be more apparent at collection time, 3) in the wild, when spermatozoa come in contact with water, there is an increase in FPM or hyper-activation of the cells for them to be able to reach and fertilize the egg before it becomes impenetrable, 4) as observed in sperm percentage motility, amphibian sperm is more resistant to hypo-osmotic shock right after dilution, while structural damage occurs after some time of exposure. This could explain the higher FPM values that we observed at collection time in the water-diluted samples and a possible effect of hormones at this time point. Additional to the hypo-osmotic injury, depletion of the sperm energy storages (ATP) might contribute to the constant decrease in the motility of *A. zeteki* spermatozoa, as it has been proposed for the loss of sperm motility of *Merlucius merlucius* [208].

After comparing sperm percentage motility and FPM between hormonal treatments and extracellular conditions by each time point and hormone treatment, a clear effect of dilution in water and temperature is observed.

Activation of freshwater fish and amphibian spermatozoa occurs when the cells come in contact with hypo-osmotic media [57,73,79,194,201]. However, the same condition that activates sperm motility and favors fertilization in these species causes cell damage and death after long-term exposures [40,69,161,229]. A study by Billard, 1978, in several species of fresh and seawater fish, reported a steady decrease in sperm motility after dilution and

activation and a detrimental effect to the morphology of the cells starting 30 sec after exposure to diluted media. Among the structural damages, the author reports swelling of the plasma membrane, formation of vesicles, deformation of the midpiece, detachment of the membrane around the head and the flagellum and coiling of the tail. These structural changes might lead to a continual loss of the motion capacity[194], thus, decreasing percentage motility and FPM over time. Christensen et al., 2004, reported that lower FPM of *X. laevis* spermatozoa in higher diluted media might be caused by hypo-osmotic damage and flagellar membrane swelling in a time dependent manner, where initial swelling does not affect motility but after longer exposure to hypo-osmotic conditions. We hypothesize that at collection time, *A. zeteki* water diluted spermatozoa become hyper-activated right upon contact with water, requiring and utilizing more ATP and external energy sources that might change depending on the effect of the hormone treatments in the seminal fluid and spermic urine, where hCG 1 IU/μl have shown to have a positive effect due to yet unknown mechanisms. Prolonged exposure to dilution (23 and 46 min after collection) might render the hyper-activated cells depleted of ATP and incapable of utilizing external sources of energy, where the first signs of morphological damage start to show, decreasing percentage motility, FPM and duration of motility in these cells. Further research on the effect of dilution on the morphology and energetics of the cells is necessary to understand how hypo-osmotic shock affects motility over time in *A. zeteki* spermatozoa. This kind of information is needed for the development of short and long-term storage protocols for the applications of ARTs in this species.

A tight dependency among osmotic shock, sperm water permeability and temperature has been established in mammalian spermatozoa, where cells exposed to lower temperatures show higher membrane integrity [230]. Osmotic shock has been related to a change in membrane permeability and structure of the lipid bilayer [73], where temperature might play a part in accelerating or delaying this detrimental changes. Higher temperatures have also been related to faster rates of energy depletion [57].

The temperature of the extracellular media directly influences sperm motility parameters [167]. Mammalian sperm, like stallion, boar or feline, is more sensitive to cold shock than amphibian sperm [11,231,232]. A decrease in temperature has been reported to favor sperm motility and duration of motility in some species of fish as well [208]. Carp spermatozoa retain motility for longer when exposed to 20 °C than to 26 °C. Mansour at al., 2010, demonstrated that *Rana temporaria* spermatozoa exposed to 0 °C remain motile for 6 h, cells kept at 4 °C show no decrease in the percentage or velocity of motility for 1 h, while those exposed to 22 °C show no motility after 45 min of exposure. Similarly, *Bufo fowleri* sperm motility decreases to ~20% after 5 h at room temperature, while samples kept at 0 °C show ≥80% motility for at least 20 h after collection [11].

Our results suggest that *A. zeteki* spermatozoa kept at 21 °C and at 4 °C show high percentage motility and FPM for at least 46 min after collection. Percentage motility, although not significant, seems to be higher for longer in samples kept at 4 °C in all hormone treatments, while percentage FPM does not show such a trend in samples exposed to 21 °C and 4 °C. Additional to the detrimental effect of dilution over time, temperature seems to have a higher impact on FPM than percentage motility in water diluted samples exposed to 21 °C. In our experimental design we did not include water

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diluted samples exposed to 4 °C because of the small sample volumes available. Future research on the effect of higher temperatures (>21 °C) on the motility parameters of spermic urine and water diluted samples, and the effect of lower temperatures in water diluted samples (4 °C) are necessary to gain wider knowledge on the effect of temperature on *A. zeteki* spermatozoa.

Morphology

We chose to analyze the effect of different extracellular environments on the morphology of *A. zeteki* spermatozoa at 23 min after collection given the small sample volumes available and the need to assess these effects after some time, but not too much, of exposure to these condition.

In the general morphology assessment, cells were classified as intact or abnormal. We considered abnormal cells those that had at least one abnormal morphology trait (acrosome, head and/or tail). Our results revealed that at 23 min after exposure to a hypo-osmotic environment, <50% of the cells remain intact. However, there was no significant difference in the percentage of cells with normal morphology among the three different extracellular conditions. Samples kept at 4 °C showed a trend of higher percentage of intact cells, although not significant, than samples kept at room temperature and in hypo-osmotic environments.

When assessing the effect of extracellular conditions on the integrity of each morphological parameter, the percentage of cells with intact acrosome or head or tail was high. Greater, but not significantly different, percentage of intact acrosomes (92.4%), heads (96.7%) and tails (88.9%) were observed in samples kept at 4 °C compared to the two other conditions. Interestingly, although we were expecting a significant decrease in

the percentage of intact morphology traits under the water-diluted condition, these samples showed >70% of the cells with intact acrosome or head or tail at 23 min after dilution. When comparing the specific morphological traits with the general morphology analysis, it is necessary to remember that cells with intact acrosome, head or tail could have had other abnormalities. Additionally, the standard deviation on the general morphology assessment indicates that there was a high variability in the responses of the samples to the different extracellular conditions. Increasing the number of samples might assist to have a better estimate of the percentage of cells with intact or abnormal morphologies under these conditions.

As mentioned above when explaining the effects of osmotic shock on amphibian spermatozoa, cells exposed to dilution suffer structural damage after some time of exposure to this condition. The time it takes for the cells to damage appears to vary among species. For example, in some species of fish, morphological damage starts to appear 30 sec after dilution [213].

Our results on the morphology assessment suggest that after 23 min of exposure to diluted media, *A. zeteki* spermatozoa have already suffered considerable structural damage. While the acrosome and head appear to be more resistant to hypo-osmotic injury at this time point, as seen in equine sperm acrosomes exposed to diluted media [233], sperm tails show higher percentage of damage upon dilution. The flagellum is the structure of the cell responsible for motility [234]. If exposure to hypo-osmotic media caused higher percentages of damage in the *A. zeteki* sperm tail, we could hypothesize that reduced percentage motility and forward progression observed at 23 and 46 min after collection could be partly due to considerable flagellar damage.

The time it takes for *A. zeteki* water-diluted spermatozoa to start showing signs of hypo-osmotic injury after dilution in water remains unknown. Further research on this and on the effect of dilution on the sperm morphology after a longer exposure is needed to understand its impact on the motility and viability of these cells. This information is necessary for the correct elaboration of ART's protocols, such as the handling time and dilution of *A. zeteki* spermatozoa in the future application of Artificial Insemination on this species.

DNA integrity

After evaluating the percentage DNA integrity of *A. zeteki* spermatozoa under three different extracellular conditions at 23 min after collection, our results indicate that exposure to water-diluted media for 23 min significantly decreases the percentage of cells with intact DNA (32.8%) compared to intact DNA from spermic urine kept at room temperature (63.2%) and at 4 °C (77.9%). Even though there was no significant difference between the percentages of intact DNA from the spermic urine samples kept at room temperature and at 4 °C, the latter showed a trend of higher percentage of DNA integrity.

Dilution in water after 23 min showed to be clearly detrimental for the DNA integrity of *A. zeteki* spermatozoa. Additionally, a decrease in the storage temperature of spermic urine from 21 °C to 4 °C seemed to delay the fragmentation of *A. zeteki* sperm DNA. Contrary to what it is observed in mammalian spermatozoa, where exposure to low temperatures >0 °C and hypo-osmotic stress increases ROS production and DNA fragmentation [233] possibly as a consequence of the internal fertilization mode, *A. zeteki* spermatozoa, like in other amphibian species [11,232], are more resistant to cold and hypo-

osmotic shock [64], where lower temperatures benefit the functionality of these cells. Our results suggest that lower storage temperatures (4 °C) might be delaying the membrane damage and the increasing production of ROS caused by dilution in the *A. zeteki* spermatozoa. However, future research on the evaluation of ROS concentrations or antioxidant enzymatic activity on *A. zeteki* spermatozoa exposed to different extracellular conditions at 23 and 46 min is needed to understand the relationship among exposure time, oxidative stress and DNA fragmentation after dilution in this species.

Since we did not assess cell viability in this study, we are not sure what percentage of cells with fragmented DNA was not viable. A parallel evaluation of cell viability and DNA integrity is necessary to understand the effect of dilution and temperature in the viability of *A. zeteki* spermatozoa with DNA fragmentation, because there is great evidence of high association among osmotic shock, oxidative stress, DNA fragmentation and finally, cell apoptosis in many cellular types, including spermatozoa [216,233,235].

Taken together, our results suggest that hormonal treatments, storage temperatures, dilution in hypo-osmotic media and the time of exposure to extracellular conditions influence *Atelopus* zeteki spermatozoa motility parameters after activation. Stimulation with the three hormone treatments we tried does not negatively affect sperm percentage motility or FPM in this species. A constant positive effect of storage at 4 °C was observed in the percentage motility, FPM, duration of motility, sperm morphology and DNA integrity. Exposure of sperm to 21 °C was not significantly detrimental for these parameters, although usually showing lower values than those observed when

samples were kept at 4 °C. Nevertheless, a clear detrimental effect of dilution over time on the motility parameters and cell integrity was evident.

This information is essential for the future development and implementation of ART's in this critically endangered species. In a previous study we already determined the best hormonal treatments for the successful stimulation of spermiation in this species. After analyzing the results of this study, we recommend that right after collection, *A. zeteki* spermatozoa should be kept in non-diluted spermic urine at 4°C for a maximum of 20 min before its use in techniques such as artificial insemination, ICSI or cryopreservation.

Chapter 4: <u>Effect of exposure to cryoprotectant agents (CPAs) before and after cryopreservation on *Atelopus zeteki* sperm viability</u>

Abstract

The IUCN prioritizes the creation of Genome Resource Banks, through the application of cryopreservation, for the conservation of endangered amphibian species. Our aim was to assess the effects of four different cryoprotectant agents (CPAs) in the motility parameters and viability of *Atelopus zeteki* spermatozoa before and after cryopreservation, to generate the necessary information for the future development of an optimal sperm cryopreservation protocol for the species. Sperm samples were obtained after stimulation with Amphiplex and samples were equilibrated for 5, 10, 15 and 20 min in four CPAs containing different concentrations of dimethylformamide (DMF) and trehalose. Cryopreservation was achieved by equilibrating the samples with supplemented CPAs for 5 and 10 min, followed by a step-wise decreased in temperature before the final plunge in LN_2. Thawing was achieved by placing the straws in water at 21 ºC for 15 sec. Spermic urine and ARS pre-diluted samples equilibrated with CPA1 and CPA3 for 5 and 10 min produced the best sperm percentage motility recovery, although still showing an average motility decrease of 60% compared to fresh samples. Cryopreservation was accomplished after supplementing these CPAs with additional agents (sucrose, sodium bicarbonate and raw egg yolk) with ARS samples cryopreserved in CPA3-REY producing the highest recovery of sperm motility and DNA integrity.

Introduction

Cryopreservation and thus, the creation of Genome Resource Banks (GRBs), are among the most powerful Assisted Reproductive Technologies (ARTs) given its extensive benefits and applications. Among these benefits are[33,35,36,209] :

- GRBs serve as insurance colonies for the existent genetic diversity.

- The different kinds of biological materials that can be stored represent an invaluable source of genetic materials.

- Cryopreserved genetic variability can be incorporated into domesticated species to improve its genetic pool and resistance to local diseases, hence supporting local economies.

- GRBs facilitate the exchange of genetic material between institutions, as specified by the Species Survival Plan, minimizing the movement of animals by instead exchanging frozen biomaterials.

- The use of cryopreserved gametes in *In-Vitro* Fertilization helps surpass mating incompatibility of genetically valuable breeding pairs.

- GRBs preserve the genetic contribution of the donor for many generations, even after the individual is dead.

- The use of cryopreserved germplasm helps minimize space problems in zoos as fewer individuals need to be part of a collection for it to maintain a healthy heterozygosity since the genetic contribution of many more can be preserved and incorporated in this collections via GRBs.

The International Union for the Conservation of Nature, IUCN, prioritizes the implementation of ARTs, especially cryobanking, for the conservation of critically endangered amphibians [33,64].

The ultimate goal of sperm cryopreservation should be preserving cellular integrity while maintaining the fertilization potential of these cells [236,237]. Freezing and thawing reduce structural integrity of spermatozoa, causing cellular membrane disruption, acrosomal damage, breakage of the flagellum, alterations in the extra/intra-cellular signaling pathways, compromising mitochondrial integrity, often rendering the cells infertile [236,238]. Development of species-specific cryopreservation protocols is necessary to meet each species cryosensitivities and mitigate the harsh effects of cryopreservation, increasing the recovery of viable cells capable of achieving fertilization [35]. Such protocols have been successfully developed for several species of mammals, including domestic economically important species such as bull, ram, stallion and goat [40,232,239-243], endangered mammalian species such as eld's deer, baird's tapir, giant panda, and cheetah [209,244-247], several species of fish [236] and for the application of assisted reproduction in humans [42,44,87,248-250]. Nevertheless, the development of such techniques in amphibian species has been limited [11]. Cryobanking has been achieved in some species of endangered amphibians such as the Puerto Rican frog, boreal toad and Mississippi gopher frog [58,251] and in some amphibian species of less concern [49-51,61,202]. Nevertheless, the progress in sperm cryopreservation of amphibians have been slow compared to other taxa [11].

Spermatozoa were the first mammalian cell type to be cryopreserved with the discovery of glycerol as a Cryoprotectant Agent (CPA) [252-254]. Amphibian and fowl

spermatozoa were also among the first successfully cryopreserved cells in the late 1930's [254,255]. Since then, many protocols have been developed for the effective cryopreservation of germplasm from a wide range of taxa, including livestock, poultry and fish [11,256].

During cryopreservation, spermatozoa are exposed to extreme non-biological conditions that include dehydration, drastic changes in temperature and contact with cytotoxic agents [40,230,232,256]. Three common consequences of exposure to these conditions are osmotic shock, cold-shock and cryoinjury, causing changes in membrane lipid composition and physical organization, oxidative stress, impaired water permeability, intracellular ice crystal formation, excessive dehydration and cytotoxicity resulting in irreversible cellular damage and low recovery of motile and viable cells [40,236,241,248,253,256]. At thawing, most of these dramatic effects should be reversed [241] and as a consequence, cryopreservation reduces sperm viability by an average of 50% [232,235]. Species-specificity plays an important role in the cellular responses to these extreme conditions since specific characteristics, such as cell morphology, the composition of the sperm membrane and seminal fluid, pH and sperm concentration vary among species and either favor or decrease the post-thaw cell viability [209].

Even though the most common parameter for the evaluation of cryopreservation success is the recovery of sperm motility, increased production of reactive oxygen species (ROS), oxidative stress and DNA base changes or fragmentation are also among the consequences of cryopreservation and their evaluation has become more popular in the recent years [232,235,238,241,250,256-258]. Even though ROS are necessary for the correct functioning of several molecular processes, its excessive production has been associated to lipid peroxidation and DNA fragmentation in several species [221]. Sperm cooling,

exposure to extenders and thawing are among the events that cause this kind of damage [40,233,249,258].

Spermatozoa are particularly susceptible to oxidative stress since lipid peroxidation occurs when ROS interact with membrane unsaturated fatty acids (very common on sperm membranes) and the limited sperm repair mechanisms leads to membrane and DNA damage [221,233]. Sperm oxidative stress, increased calcium concentrations and endonuclease activity, and DNA fragmentation have been associated with a decrease in motility, fertility and impaired embryonic development [83,238,259,260]. However, DNA fragmentation can be present in cells displaying other "normal" parameters, such as motility, capable of fertilizing eggs, causing post-fertilization problems that lead to mutations, abortions and embryo developmental problems [248,257,258]. The evaluation of these parameters, as well as finding an optimal CPA that provides protection against it, is essential for the recovery of viable cells after cryopreservation and their subsequent use in ARTs. Trout spermatozoa cryopreserved with DMSO and LDL (purified from egg yolk) showed increased membrane and DNA integrity in comparison to DMSO and pure egg yolk, suggesting that the LDL fraction of egg yolk confers this kind of protection [258]. Cabrita et al., 2005 also found an increased percentage of DNA fragmentation in cryopreserved trout spermatozoa while no difference was found between fresh and cryopreserved sea bream sperm DNA. Cryopreservation and the posterior thawing of human and stallion spermatozoa have been related to an increased production in ROS and DNA fragmentation [221]. Species-specific chromatin packaging influences the DNA susceptibility to fragmentation and may account for the differences observed among taxa [83].

Successful recovery of viable spermatozoa depends on finding the correct equilibrium among the cooling, super cooling and freezing temperature rates and the CPA's type and concentration to avoid excessive cell dehydration before reaching safe temperatures, limiting cytotoxicity and intracellular ice formation [230,253]. The most commonly used cryoprotectants usually contain a mixture of penetrating and non-penetrating CPA's [32,261]. While the main role of CPAs is to reduce ice formation during the freezing process, penetrating CPAs, those that cross the cell membrane such as glycerol, dimethyl sulphoxide (DMSO), methanol (ME), propylene glycol (PG) and ethylene glycol (EG) tend to have a cytotoxic effect, negatively interacting with ultrastructural elements, probably changing the viscosity of the cytoplasm, altering intracellular processes and also causing osmotic shock [241,248,253,262]. The effects of these CPAs on cell viability are dependent on temperature, CPA permeability and exposure time [232,248,253]. If the cooling rate is too fast, there is not enough time for water to exit the cells resulting in intracellular ice formation and cell death, while if the rate is too slow, cells will undergo hyper-osmotic and cytotoxic shock [209,263].

Additionally, the species sensitivity to cold-shock plays an important role before and during cryopreservation. In some species, such as boars, sperm exposed to a reduction in temperature from 30 °C to 0 °C right after ejaculation undergo a dramatic decline in viability, possibly due to a phase change in the membrane lipids, alteration of other membrane components and cytoskeletal elements, compromising cell survival [232,241]. Furthermore, CPAs have an effect on sperm water permeability by interacting with membrane components, sometimes modifying water transport proteins and blocking its

passage, resulting in an impaired water movement [42,263]. Membrane permeability during cryopreservation is also influenced by temperature and ice formation [40].

Glycerol has been widely used as a penetrating CPA since its cryopreservative characteristics were discovered, however its effectiveness varies among species [59,209,232,242,256,264]. The toxic effect, high viscosity and slower permeability of glycerol may contribute to this differences in cryopreservation efficacy [239,263-266]. Several other penetrating CPAs have been recently more commonly used, also showing species-specificity in their efficiency as CPAs [239,267]. Amides such as formamide, methylformamide, dimethylformamide or acetmaide have a smaller molecular weight than glycerol, greater water solubility and membrane permeability, which may contribute to decrease cytotoxic and osmotic shock [41,239,265,267]. Dimethylformamide (DMF) has proven very effective for the cryopreservation of stallion spermatozoa compared to glycerol and other amides, improving sperm motility, viability and fertilization capacity [239]. Contrary to the effects on stallion spermatozoa, DMF has rather a negative influence on the motility parameters of goat spermatozoa when compared to glycerol, however fertilization capacity is somewhat retained [265]. A study by Varela Junior at al., 2012, demonstrated the positive effects of DMF in tambaqui (*Colossoma macropomum*) spermatozoa, increasing percentage motility, membrane integrity, mitochondrial function and DNA integrity, when compared to treatments with glycerol, DMSO, dimethylacetamide and methylformamide. DMF in combination with trehalose resulted in the successful sperm cryopreservation of two endangered species of amphibians: the boreal and the Mississippi gopher frog, where recovery of motility exceeded 50% [268].

The role of non-penetrating CPAs, those that do not cross the plasma membrane, such as sugars, proteins or lipids, is the stabilization of the plasma membrane and attenuation of cold and osmotic shock [269,270]. Sugars (i.e. sucrose, raffinose, glucose, trehalose and fructose) and egg yolk are among the most popular non-penetrating CPA's [32]. Sugars are believed to act by increasing the proportion of the unfrozen water fraction while reducing the concentrations of salts [241]. Trehalose acts by interacting with cellular proteins and membrane lipids, probably increasing membrane fluidity [241,269]. The positive effects of trehalose, used as the non-permeating CPA fraction in combination with a variety of permeating CPAs, have been observed in several species. Mouse sperm post-thaw membrane integrity increases when trehalose and glycerol, but not raffinose and glycerol, are used as CPA [271]. The use of trehalose reduces the cytotoxicity caused by acetamide in stallion sperm [239]. Goat sperm percentage motility, percentage intact acrosome and membrane fluidity increase in the presence of high concentrations of trehalose compared to sucrose [269].

Although egg yolk has proven effective for membrane stabilization and protection against cold-shock through its low-density lipoprotein (LDL) component and phospholipids, the exact mechanism by which this protection is conferred is still unknown [241,243,256]. Addition of egg yolk to CPAs has resulted beneficial for the cryopreservation of several species. A study by Silva et al., 2002, in dog spermatozoa demonstrated that egg yolk in combination with glycerol and Tris buffer favored sperm motility parameters. Osmotic tolerance of mouse spermatozoa improves with the addition of egg yolk [43]. Studies on ram spermatozoa have demonstrated the effect of egg yolk in preventing flagellar and mid-piece bending [241]. Optimal CPAs containing egg yolk have

been developed for other species such as the Giant Panda, black-footed ferret and Indian rhinoceros [209]. Sperm from yellow perch shows improved fertilization capacity when cryopreserved with DMSO and egg yolk [272], while rainbow trout sperm quality increases when using egg yolk in combination with sucrose [273]. Egg yolk can also be detrimental for cryopreserved sperm viability and its effects appear to be species-specific as well [274]. Asp spermatozoa shows post-thaw reduced viability when cryopreserved with CPAs containing egg yolk compared to the results obtained using the same CPAs without egg yolk [274]. Studies on Danube bleak spermatozoa revealed that pre-freezing exposure and cryopreservation with the addition of egg yolk reduced sperm motility and velocity in this species compared to other non-penetrating CPAs [273].

The development of amphibian sperm cryobanking techniques has been slow compared to other economically important or more "charismatic" endangered species, but in the recent decades, due to the current amphibian crisis where ~200 species have been lost and many more are on the brink of extinction, some progress have been reported [11,61,275]. The application of sperm cryopreservation and the recovery rate of viable cells in amphibian species seem to be as highly variable and species-specific as in other taxa. Amphibian sperm cryopreservation was originally accomplished through the use of testicular spermatozoa, where whole testes are removed after euthanasia [61,65,275]. Even though this technique has been used in some species, its application on the conservation plans of endangered species is controversial since it involves the killing of highly valuable specimens [11,61]. Some studies have reported successful recovery of motility from testicular cryopreserved spermatozoa using DMSO or glycerol in combination with sucrose or egg yolk as CPAs in species such as the cane toad (*Bufo marinus*), the

European common frog (*Rana temporaria*), the wood frog (*Rana sylvatica*), the leopard frog (*Lithobates pipiens*), the American toad (*Anaxyrus americanus*), African clawed frog (*Xenopus laevis*) and the Puerto Rican frog (*Eleutherodactylus coqui*) [49,50,56,57,59,112].

Freezing hormonally induced spermic urine has proven more difficult than testicular sperm [275] thus available reports are limited. A study by Hopkins et al., 2007, on *Rana pipiens* spermic urine cryopreservation showed that a CPA mainly composed by glycerol, glucose and egg yolk yielded higher percentages of viable sperm than other CPAs containing DMSO and sucrose or glycerol and sucrose. Another report by Shishova et al., 2011, on successful cryopreservation of *Rana temporaria* spermic urine, showed higher recovery of sperm motility, membrane integrity, fertilization and larval survival when DMF was used as CPA compared to DMSO, glycerol, DMSO/egg yolk and MIS/DMSO. Langhorne et al., 2013 also reported successful cryopreservation of *Anaxyrus boreas boreas* and *Lithobates sevosa* spermic urine with >60% recovered motility using DMF and trehalose as CPA.

Our hypothesis was different CPAs affect *A. zeteki* sperm viability and that cryopreservation of spermic urine affects subsequent sperm motility parameters and DNA integrity. Our specific aims were to assess the effects of exposure to different cryoprotectants before and after freezing on: 1) sperm motility and Forward Progressive Motility (FPM) and 2) DNA integrity. The main purpose of the study was to gain some knowledge on the effects of cryopreservation on *A. zeteki* sperm functionality and quality for the future development of successful cryopreservation protocols and the creation of a Genome Resource Bank for the species.

Materials and Methods

Animals and Approvals

Thirty adult males *Atelopus zeteki* (>2 year old, average weight of 3.81±0.41 g) were obtained from the Species Survival Plan (SSP) population from the Maryland Zoo in Baltimore, MD. Animal Use Protocols were submitted for the Institutional Animal Care and Use Committee (IACUC) approval from the University of Maryland, the Smithsonian National Zoological Park (SNZP) in Washington, D. C., and Maryland Zoo in Baltimore, MD. After obtaining IACUC approvals, the animals were transferred from the Maryland Zoo to the SNZP and were placed 30 days under quarantine. One individual died during quarantine and two more died in the next two years.

Six terraria (Exo-Terra Terrarium, 24"x18"x24") were adapted and designed to accommodate 5 males per tank. Each tank was fitted with live potted plants and live moss as substrate. The recommended humidity in the tanks (60% to 90%) was achieved with the use of an automated misting system that works with reverse osmosis filtered water and that was set up to mist once every hour for 2 minutes. Excess water was discarded utilizing a flow through drainage system. Temperature ranged between 20-22 °C with the use of a window unit air conditioner. All frog terraria were supplied with a UVb Light System, (Zoomed's 10.0 T5 High Output Bulbs) to avoid hypovitaminosis D_3 and help with proper bone growth [141].

Frogs were fed 4 times per week. Food items included; 30 one-week-old crickets dusted with a calcium carbonate powder containing vitamin D_3, 30 wingless fruit flies and bean beetles. Items were fed on a rotating basis.

For identification purposes, 4 photographs of each animal were taken to distinguish males utilizing their spot patterns and an alpha-numeric code was assigned to each as follows: PGF01 to PGF30, where PGF stands for Panamanian Golden Frog.

Hormone Stimulation and Sperm Collection

Hormonal stimulation was administered via intracoelomic injection with the help of a Veterinarian from the National Zoo [11,62]. Treatments were carried out once a week for twelve weeks by stimulating groups of four randomly selected males with 40 μl of Amphiplex [0.4 μg of GnRH-A [des-Gly[10], D-Ala[6], Pro-NHEt[9]] (Bachem Americas Inc., Torrance, CA, USA) and 10 μg of Metoclopramide hydrochloride (Sigma-Aldrich Corporation, St. Louis, MO, USA)] [30].

After injections, males were placed inside ventilated plastic containers with water soaked paper towel on the bottom and covered from light to reduce stress. Spermic urine was collected 3 hours post-injection by gently inserting a small catheter [0.023" I.D. x 0.039" O.D., Micro Medical Tubing, 85 Durometer Vinyl (Scientific Commodities Inc., Lake Havasu City, AZ, USA)] in the cloacae [65].

One sperm sample per male was collected. For each collection, samples were divided into 2 aliquots (conditions) as follows: SU (non-diluted spermic urine) and ARS, a 1:1 dilution of spermic urine in Amphibian Ringer Solution (Carolina Biological Supply Company, Burlington, NC, USA). Samples were kept at 4 °C until processed.

Sperm processing and analysis

Basic metrics were utilized to define the general characteristics of *A. zeteki's* spermic urine. Total volume of excretion was determined with the use of a micropipette. Sperm

concentration was assessed with the use of a hemocytometer; 10 μl of spermic urine were added to the chamber and counted following the 5 square rule.

Percentage of sperm motility was determined by counting all the cells with flagellar movement from a total of 100 cells analyzed under an Olympus BX41 microscope at a 400X magnification. Percentage forward progresive motility (FPM) was obtained by counting all the cells expressing forward motility relative to the 100 cells that were expressing any kind of flagellar movement.

Percentage motility and FPM were determined before and after exposure to cryoprotectants (CPAs).

CPAs, exposure times and cryopreservation

In this study we wanted to evaluate the effect of exposure to two conditions [spermic urine (SU) or amphibian ringer solution (ARS)], different cryoprotectant agents and concentrations and times on the percentage motility, forward progressive motility and DNA integrity of Panamanian golden frog (*Atelopus zeteki*) spermatozoa before and after cryopreservation.

Preliminary cryopreservation tests in our laboratory where we attempted using different combinations of penetrating and non-penetrating CPAs in *A. zeteki* spermic urine and testicular spermatozoa resulted in no successful recovery of motile cells (data not shown). These CPAs included penetrating agents such as DMSO, glycerol, formamide and methylformamide, along with non-penetrating agents such as trehalose, sucrose and egg yolk. Recovery of motile (viable) cells was only accomplished with the use of CPAs containing DMF and trehalose; this is the reason why all of the CPAs used in this study contained the same basic components with some adjustments.

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The modifications made in the last two trials in terms of CPA composition and addition of ramps resulted from the fact that we recovered no motile cells after the first and second cryopreservation trials.

Cryotoxicity Trials

We evaluated the effect of 5, 10, 15 and 20 min of exposure to 4 cryoprotectants on the percentage motility and FPM of *A. zeteki* spermatozoa. All CPAs contained different concentrations of N,N-Dimethylformamide (DMF) and trehalose (Sigma-Aldrich Corporation, St. Louis, MO, USA), as follows:

- **CPA1:** final concentration of 10% DMF and 10% trehalose.

- **CPA2:** final concentration of 10% DMF and 5% trehalose.

- **CPA3:** final concentration of 5% DMF and 10% trehalose.

- **CPA4:** final concentration of 5% DMF and 5% trehalose.

Spermatozoa collected from 4 males were exposed to each CPA as spermic urine alone (SU) or ARS by each exposure time as shown in Figure 24. The CPAs osmolalities were obtained with the use of an osmometer (Vapro Osmometer 5520, Wescor, Inc, South Logan, Utah, USA).
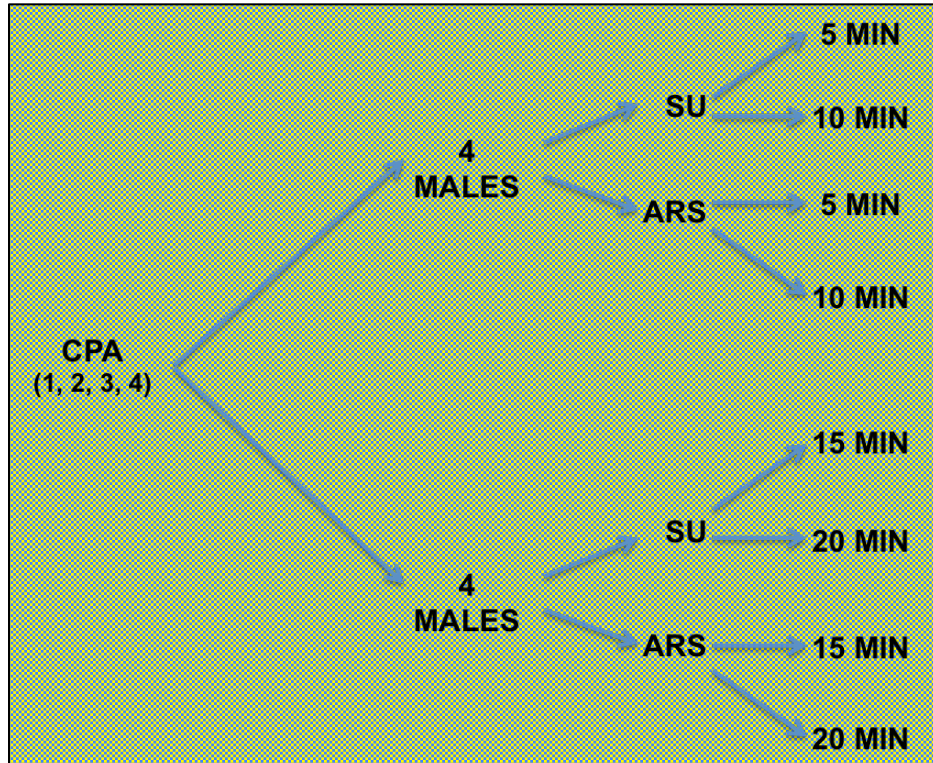
Figure 24. Cryotoxicity trials. The figure shows the number of CPAs (4), males per CPA (8), conditions (SU/ARS) and exposure times for each sample.

Spermic urine (SU) and ARS samples were diluted 1:1 in each CPA and kept at 4 °C for equilibration during 5, 10, 15 and 20 min. After each equilibration time, samples were diluted 1:5 in distilled water for the activation of sperm motility. Percentage motility and FPM were recorded as explained above.

After assessing the effects of exposure to the different CPAs and exposure times on the motility parameters of *A. zeteki* spermatozoa, CPAs and exposure times that reduced pre-freezing sperm motility were excluded and we decided to continue the cryopreservation trials using CPA1 and CPA3 equilibrated for 5 and 10 min at 4 °C.

Cryopreservation Trials

1. **CPA1 and CPA3 exposed for 5 and 10 min to LN₂ vapor**

   Before cryopreservation, two samples from different males, each divided into SU and ARS aliquots, for each CPA were equilibrated for 5 and 10 min at 4 °C, as explained above. After equilibration, samples were immediately loaded into 0.25 cc, clear, gamma irradiated cryopreservation straws (Mai Animal Health, Elmwood, WI, USA), heat-sealed, exposed for 5 min to LN₂ vapor 10 cm above the liquid surface (-60 °C) and finally plunged into LN₂ (-198 °C). Temperatures were measured with a thermocouple (Omega Engineering, Inc., Stamford, CT, USA). Samples were maintained in LN₂ for at least one hour prior to thawing by immersion in tab water at room temperature (21 °C) for 15 seconds. Percentage motility and FPM were recorded as explained above.

2. **CAP1 and CPA3 with TEST yolk buffer (TYB) exposed for 3 min to LN₂ vapor 10 and 5 cm above the liquid surface**

   In the second trial we wanted to assess the effect of a commercial egg yolk based buffer added to the CPAs utilized in the first trial. TEST yolk buffer [non-disclosed commercial formulation: TES, Tris, Sodium Citrate, fructose, 20% v/v heat-inactivated egg yolk protein, 10 μg/ml gentamicin sulfate and 12% v/v glycerol] (Irvine Scientific, Santa Ana, CA, USA) is a commonly used extender for the cryopreservation of spermatozoa. The composition of the modified CPAs follows:

- **CPA1-TYB:** final concentration of 10% DMF, 10% trehalose and 10% TYB.

- **CPA3-TYB:** final concentration of 5% DMF, 10% trehalose and 10% TYB.

The CPAs osmolalities were obtained with the use of an osmometer (Vapro Osmometer 5520, Wescor, Inc, South Logan, Utah, USA).

Before cryopreservation, two samples from different males, each divided into SU and ARS aliquots, for each CPA were equilibrated for 5 and 10 min at 4 °C, as explained above. After equilibration, samples were immediately loaded into 0.25 cc, clear, gamma irradiated cryopreservation straws (Mai Animal Health, Elmwood, WI, USA), heat-sealed, exposed for 3 min/ramp to LN_2 vapor 10 cm (-60 °C) and 5 cm (-95 °C) above the liquid surface and finally plunged into LN_2 (-198 °C). Temperatures were measured with a thermocouple (Omega Engineering, Inc., Stamford, CT, USA). Samples were maintained in LN_2 for at least one hour prior to thawing by immersion in tab water at room temperature (21 °C) for 15 seconds. Percentage motility and FPM were recorded as explained above.

3. **CAP1 and CPA3 with raw yolk (REY) exposed for 3 min to LN_2 vapor 15, 10 and 5 cm above the liquid surface**

In the third trial we wanted to assess the effect of raw egg yolk added to the CPAs utilized in the first trial. Raw egg yolk has been successfully used in the cryopreservation of spermatozoa from many species, including fish and amphibian, since the its lipids and proteins content provide membrane protection

to the cells [32,51,236,248,276]. The egg yolk stock solution (REY) was prepared following Browne and Zippel, 2007, recommendations with some modifications. Five hundred μl of chicken egg yolk were diluted 1:1 in distilled water and sonicated for 1 min. Five hundred μl of this initial solution were diluted 1:5 in distilled water and sonicated again for 1 min.

The composition of the modified CPAs follows:

- **CPA1-REY:** final concentration of 10% DMF, 10% trehalose, 10% REY, 800 mM sucrose and 20 mM sodium bicarbonate.

- **CPA3-REY:** final concentration of 5% DMF, 10% trehalose and 10% REY, 800 mM sucrose and 20 mM sodium bicarbonate.

Before cryopreservation, four samples from different males, each divided into SU and ARS aliquots, for each CPA were equilibrated for 5 and 10 min at 4 °C, as explained above. After equilibration, samples were immediately loaded into 0.25 cc, clear, gamma irradiated cryopreservation straws (Mai Animal Health, Elmwood, WI, USA) and heat-sealed. In this trial, the samples were exposed for 3 min/ramp to LN_2 vapor 9 cm (-55 °C), 5 cm (-95.7 °C) and 3 cm (-181 °C) above the liquid surface and finally plunged into LN_2 (-198 °C). Temperatures were measured with a thermocouple (Omega Engineering, Inc., Stamford, CT, USA). Samples were maintained in LN_2 for at least one hour prior to thawing by immersion in tab water at room temperature (21 °C) for 15 seconds. Percentage motility and FPM were recorded as explained above.

The osmolality of all CPAs was obtained with the use of an osmometer (Vapro Osmometer 5520, Wescor, Inc, South Logan, Utah, USA).

DNA integrity

The DNA integrity assessment was made with the use of the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique (DeadEnd™ Colorimetric TUNEL System, Promega Corporation, Madison, WI, USA), with an in situ cell detection kit, following the manufacturer's instructions. Ejaculated spermatozoa used in this assay were previously fixed with 4% paraformaldehyde and kept at 4°C until analyzed. Slides were prepared by smearing the samples and letting them air-dry. Slides were then gently rinsed twice with PBS for 5 minutes. Cells were permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature. Slides were again gently rinsed twice with PBS for 5 minutes followed by incubation with the equilibration buffer for 10 minutes at room temperature. Then, 100 µl of previously prepared rTdT reaction mix were added to the slides. After covering with plastic coverslips, slides were incubated at 37°C for 60 minutes in a humidified chamber. The reaction was terminated by removing the coverslips and adding 200 µl of 2X SCC to each slide using a pipette, incubating them for 15 minutes at room temperature. Slides were rinsed twice with PBS for 5 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 5 minutes. Slides were again rinsed twice with PBS for 5 minutes followed by incubation with 200 µl of Streptavidin Horseradish Peroxidase for 30 minutes. Slides were rinsed twice with PBS for 5 minutes and developed with 3,3′-diaminobenzidine. Slides were mounted with Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, PA, USA) and analyzed under an Olympus BX41 microscope. One hundred cells were counted per slide and classified as intact or fragmented, those with DNA fragmentation were stained dark brown.

Statistical analysis

All data from the cryotoxicity and cryopreservation trials was analysed in R programing language (R Core Team, 2015). The lmer function [lme4 package [277]] was used to perform linear regression utilizing mixed effects models. Sperm percentage motility, percentage forward progressive motility and percentage intact DNA were the response variables evaluated in the analysis. Visual assessment indicated the data was normally distributed. No data points were eliminated. For each of these variables, a set of 6 linear mixed models was created. For all models, individuals functioned as the random effect since some of the individuals were sampled more than once and to account for individual differences. Fixed variables considered in the models were fresh sperm percentage motility, cryoprotectant agent (CPA), condition (SU/ARS) and exposure time.

To select the most parsimonious model for the data, we used the residual maximum likelihood and the Akaike's information criterion (AIC) [278]. Within each set of models, the one with the lowest AIC value was considered the best fit for the data. Estimates were calculated from the selected model in which a given variable appeared. When models were run with and without the random effect, the AIC value indicated the random effect should be included in the final models. In case of existent interactions, multiple comparisons were performed. Final models for the cryotoxicity and cryopreservation trials by response variable are described in Table 4. All statistical analysis were considered significantly different at p<0.05.

Table 4. Final models used for the data analysis by trial. Response variables were assessed in each candidate model. Table shows the fixed effect variables (CPA, condition and exposure time) used per candidate model. Akaike's information criterion (AIC) and the difference between the top-ranked model and the second-ranked model (AIC difference) are shown. All models include individuals as random effect.

TRIAL	RESPONSE VARIABLE	MODEL USED	AIC	AIC DIFFERENCE
Cryotoxicity	% Motility	CONDITION + CPA	816.1	4.8
	% FPM	CPA*TIME	703.3	5.9
Cryopreservation	% Motility	CONDITION	193.1	1.2
	% FPM	CONDITION	259.2	5.3
	% Intact DNA	CPA	205.2	5.6

Results

Table 5 shows the results of effect estimates, response variables at each level and

their respective p value by each trial for all the final models selected in the analysis. .

Table 5. Effect estimates for each predictor variable and its corresponding levels included in final models for each trial. Table shows trial, response variables, predictor variables and levels, effect estimates and p values.

Trial	Response variable	Predictor variable	Level	Effect estimate	p value
Cryotoxicity	% Sperm motility	Condition	SU	2.9020	0.010369
		CPA	CPA1	19.0780	8.84E-09
			CPA2	-16.4890	3.46E-14
			CPA3	-9.5890	0.000763
			CPA4	-17.5280	1.08E-09
	% FPM	CPA*Time	CPA1/5 min	18.4740	1.69E-10
			CPA2/5 min	-15.1230	3.25E-07
			CPA3/5 min	-9.1740	0.00761
			CPA4/5 min	-13.9960	2.04E-05
			CPA1/10 min	-5.0000	0.04573
			CPA1/15 min	-14.4270	5.73E-05
			CPA1/20 min	-16.0520	9.38E-06
			CPA2/15 min	13.0460	1.28E-03
			CPA2/20 min	13.0460	1.28E-03
			CPA3/15 min	16.3190	1.07E-03
			CPA3/20 min	13.8190	5.24E-03
			CPA4/10 min	7.8890	4.39E-02
			CPA4/15 min	10.5290	3.07E-02
			CPA4/20 min	14.3090	3.01E-03
Cryopreservation	% Sperm motility	Condition	SU	-5.9290	0.085225
			ARS	10.5710	0.000662
	% FPM	Condition	SU	-27.6430	0.02111
			ARS	39.0710	0.00182
	% Intact DNA	CPA	CPA1-REY	22.5750	0.000254
			CPA3-REY	14.4870	0.016272

Osmolalities of CPAs as follow: CPA1 1393 mOsm/kg, CPA2 1483 mOsm/kg, CPA3 1040 mOsm/kg, CPA4 1387 mOsm/kg, CPA1-REY 1582 mOsm/kg, and CPA3-REY 1237 mOsm/kg.

Cryotoxicity Trials

For the cryotoxicity trials, condition [spermic urine (SU) and amphibian ringer solution (ARS)] and CPAs were the variables with the greater effect on percentage motility, while an interaction between CPA and time had the greater effect over percentage forward progressive motility. For percentage motility, CPA1 showed higher motility followed by CPA3 (Fig 25), regardless of exposure time. Samples kept in spermic urine showed higher percentage motility compared to those kept in Amphibian Ringer Solution (ARS) (empirical mean ± SEM: CPA1/SU 42.75 ± 7.80%; CPA3/SU 31.88 ± 7.05%; CPA1/ARS 32.69 ± 7.61%; CPA3/ARS 28.29 ± 5.99%).
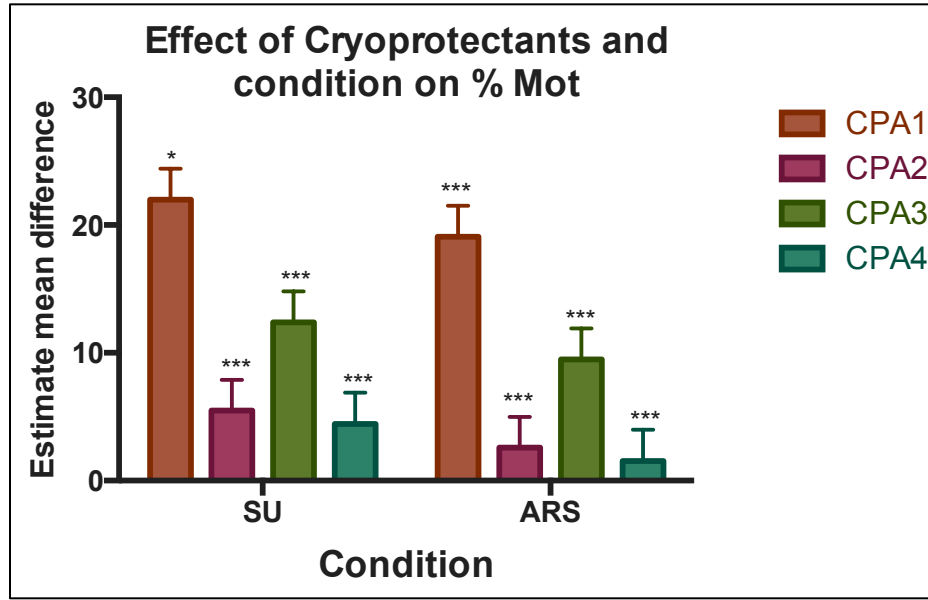
Figure 25. Effect of CPAs and condition [spermic urine (SU) and amphibian ringer solution (ARS)] in *A. zeteki* sperm percentage motility after equilibration. Estimate mean difference from the intercept ± SEM, n=27. Tukey's multiple comparison test results are shown as asterisks above the columns. Significance codes: ***(0), **(0.001), *(0.01).

For percentage FPM an interaction between CPA and exposure time revealed that the effect of CPAs in FPM depended on the time the cells were exposed to them. CPA1 equilibrated at 4 °C showed the highest significant pot-exposure FPM when cells were equilibrated for 5 and 10 min, followed by CPA3 (empirical mean ± SEM: CPA1/5 min 38.5 ± 9.78%; CPA3/5 min 31.75 ± 11.61%; CPA1/10 min 41.13 ± 7.95%; CPA3/10 min 35.25 ± 10.27%). Nevertheless, exposure to CPA3 showed significantly higher post-exposure FPM when samples were equilibrated for 15 and 20 min at 4 °C (empirical mean ± SEM: CPA3/15 min 40.38 ± 13.19%; CPA3/20 min 23.5 ± 11.71%) (Figure 26).
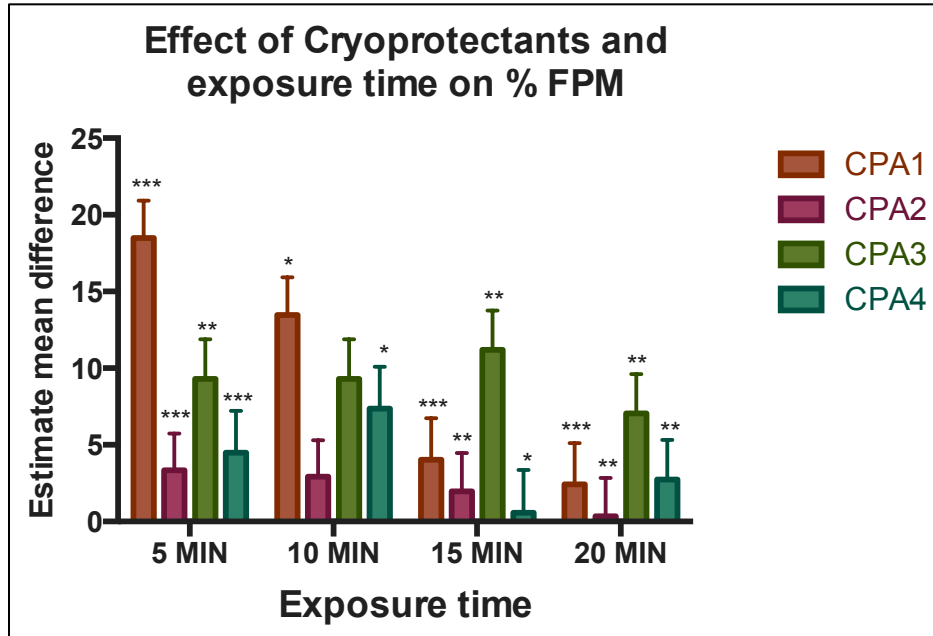
Figure 26. Effect of CPAs and exposure time in *A. zeteki* sperm percentage forward progressive motility during equilibration. Estimate mean difference from the intercept ± SEM, n=27. Tukey's multiple comparison test results are shown as asterisks above the columns. Significance codes: ***(0), **(0.001), *(0.01).

Cryopreservation Trials

For the cryopreservation trials 1 and 2, no recovery of motility was observed in any of the samples cryopreserved with CPA1 and CPA3 alone or CPA1-TYB and CPA3-TYB. Nevertheless, motility was recovered in the cryopreservation trial 3 (CPA1-REY and CPA3-REY). Our results show that condition, regardless CPA and exposure time, was the variable that had the greater effect on post-thaw *A. zeteki* sperm percentage motility and percentage forward progressive motility. Samples kept in ARS showed higher percentage motility after freezing that those kept in SU (empirical mean ± SEM: SU 4.64 ± 1.68%; ARS 10.57 ± 2.96%) (Fig 27). Similarly, samples kept in ARS showed higher

percentage FPM than those kept in spermic urine (empirical mean ± SEM: SU 11.43 ± 7.24%; ARS 39.07 ± 10.12%) (Fig 28).
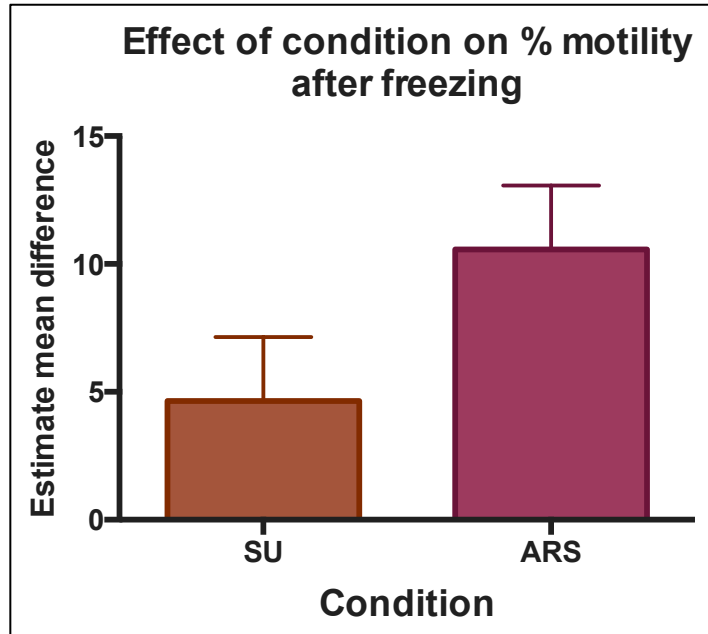
Figure 27. Effect of condition in *A. zeteki* sperm percentage motility after cryopreservation. Estimate mean difference from the intercept ± SEM, n=16. Tukey's multiple comparison test results are shown as asterisks above the columns. Significance codes: ***(0), **(0.001), *(0.01).
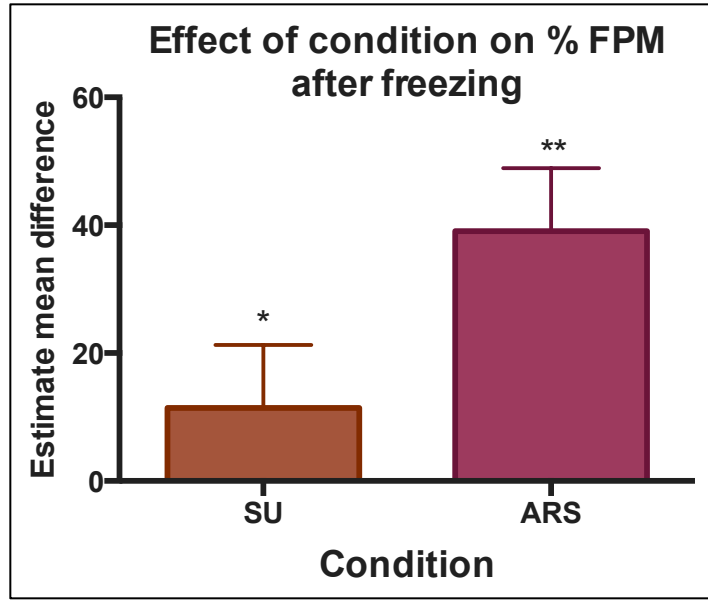
Figure 28. Effect of condition in *A. zeteki* sperm percentage forward progressive motility after cryopreservation. Estimate mean difference from the intercept ± SEM, n=16. Tukey's multiple comparison test results are shown as asterisks above the columns. Significance codes: ***(0), **(0.001), *(0.01).

Our results also showed that CPAs had the greatest effect on the preservation of DNA integrity, regardless condition and exposure time. CPA3-REY showed higher percentage of cells with intact DNA than those cryopreserved in CPA1-REY (empirical mean ± SEM: CPA1-REY 21.58 ± 1.97%; CPA3-REY 32.12 ± 4.97%) (Fig 29).
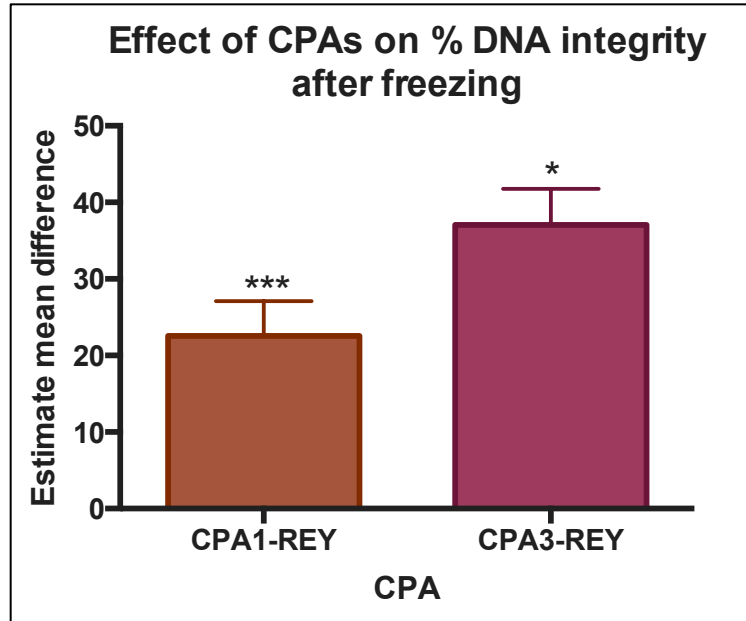
Figure 29. Effect of CPAs in *A. zeteki* sperm percentage DNA integrity after cryopreservation. Estimate mean difference from the intercept ± SEM, n=16. Tukey's multiple comparison test results are shown as asterisks above the columns. Significance codes: ***(0), **(0.001), *(0.01).

Discussion

The amphibian extinction crisis, where an estimated 40% (~2,400) of all amphibian species are under some degree of extinction risk [1,148], has created an immediate and urgent need in the scientific community for the implementation of ex-*situ* conservation plans, including the creation of captive breeding programs and the application of ARTs [11,33,52,63,64,94,279-282]. Panamanian golden frogs are considered among the most charismatic critically endangered amphibian species [20] and there is little to no knowledge on the implementation of ARTs in this species. To our knowledge, this is the first report on

Atelopus zeteki sperm cryopreservation and a first step in the improvement of such an important technique for this species for the future formation of a Genome Resource Bank.

Our results showed that equilibration decreases motility parameters in an average of 60% compared to fresh spermatozoa (average >90%). As part of the cryopreservation process, cells are exposed to cryoprotectants (CPAs) before freezing for osmotic equilibration and some studies point out that during this process, and again at thawing, is when most of the cell damage occurs [245]. Addition of CPAs to sperm samples creates an osmotic gradient to which the cells respond by losing water and shrinking [235]. Pre-freezing exposure to CPAs, necessary for the CPAs to penetrate the cells and confer protection during the actual freezing in some species, impacts some sperm parameters such as membrane and acrosomal integrity and thus motility and fertilizing capacity, mainly due to osmotic shock and CPA toxicity [261,263,272]. Some of the factors contributing to cell damage (or not) are the CPA's cell permeability and molecular weight and the change in its permeability due to temperature [41,239,261]. The reduction we observed in sperm motility might be caused by osmotic shock after the initial exposure to CPAs or/and by dilution after exposure. Some species, like the cheetah and the Nile tilapia, are more resistant than others to sperm damage at the initial exposure to CPAs during the equilibration process [209,245,272]. Even though amphibian sperm is known to be somewhat resistant to osmotic and cold shock [61], damage still occurs.

The response of cells to CPA exposure seems to be species specific since it is associated with particular reproductive modes, sperm membrane, morphological and physiological characteristics that differ from species to species [57,209,235]. Spermatozoa from anurans with external fertilization are immotile in the testes and acquire motility

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upon contact with urine (hypo-osmotic environment) [11,32,76]. The osmolality of our CPAs is around 5 times higher than that of the testes and 19 times higher than that of spermic urine, so inactive spermatozoa that gained motility after dilution in urine was inactivated again after exposure to CPAs and activated one last time after dilution in water for reactivation [65]. As amphibian sperm is highly susceptible to mechanical stress [55,61], CPAs were not removed from the samples by centrifugation at reactivation. Motility was achieved by diluting the sample in water, therefore creating additional osmotic stress on the cells. Additionally, the osmolality of ARS is similar to that of the testes (300 mOsm/kg), thus samples that were diluted in ARS previous to CPA addition experienced a step-wise increase in the osmolality. Perhaps these activation-inactivation-reactivation cycles might have caused cell damage and the drop in *A. zeteki* sperm post-exposure motility parameters. Nevertheless, different species might respond differently to these cycles. In the common frog, *Rana temporaria*, equilibration for 40 min in 5% glycerol, 10% DMSO and 10% methanol has no negative effect on sperm motility [57]. In this same species, Shishova et al., 2011, demonstrated that equilibration for 10 min at 4 °C with subsequent cryopreservation in a CPA containing DMF produced greater sperm motility and membrane integrity than equilibration and cryopreservation with CPAs containing different combinations of DMSO, sucrose, egg yolk and glycerol.

Many cryopreservation protocols include an equilibration step before freezing to minimize toxic and osmotic damage [209] but finding the adequate exposure time and temperature for a species is essential to facilitate osmotic equilibrium with the smaller proportion of cell damage. Stallion spermatozoa equilibrated for 1 hour at 5 °C prior to freezing in a CPA containing DMF shows higher post-thaw motility than non-

equilibrated samples [239]. Similarly, the optimal cryopreservation protocols for Tammar wallaby and Brushtail possum spermatozoa require equilibration for 5 min in 17.5% glycerol and PBS or 7.5% DMSO, 10% glycerol CPAs [209]. Equilibration for at least 4 hours is necessary for the successful cryopreservation of buffalo spermatozoa [283]. Li et al, 2010, found that common carp spermatozoa motility is negatively correlated to equilibration time in DMSO while membrane integrity is not compromised. Similarly, European catfish spermatozoa show higher fertilization potential when equilibrated for 5 min in DMSO, compared to equilibration for 0 and 20 min [284].

According to our models, condition and CPAs, while not exposure time, had an overall effect on *A. zeteki* sperm percentage motility during the equilibration process. Equilibration in CPA1 and CPA3 of samples kept in spermic urine at 4 °C, regardless of exposure time, presented the highest post-exposure percentage motility. Both cryoprotectants contain 10% trehalose differing in the DMF concentration (CPA1 10%, CPA3 5%). While CPA2 and CPA4 contain both 5% trehalose and the same concentrations of DMF as CPA1 and CPA3, respectively, the post-exposure percentage motility of these CPAs was very poor. This might indicate that 10% trehalose, along with DMF, is conferring some kind of protection to *A. zeteki* spermatozoa during the equilibration process. It is worth to mention that even though spermic urine samples showed significantly higher percentage motility than ARS samples, these still showed similar recovery rates as SU, indicating that pre-exposure dilution in ARS is not detrimental to *A. zeteki* equilibrated spermatozoa. Dimethylformamide has been previously used successfully for the cryopreservation of spermatozoa in several species of mammals, fish and amphibians [239,265,267,268]. Its lower molecular weight and faster cell

permeability compared to other penetrating CPAs seem to mitigate the damage caused by equilibration and cryopreservation [41,239,265,267]. The positive effects of using trehalose as a non-penetrating CPA have also been shown in several species, where an increased cell protection and reduction of CPA toxicity is observed [239,269,271]. The particular combination of trehalose and DMF has been used successfully for cryopreservation of amphibian spermatozoa. Langhorne et al., 2013, effectively recovered >60% motility from boreal toad and Mississippi gopher frog spermatozoa after equilibration for 10 min 4 °C followed by cryopreservation in a CPA containing 10% trehalose and 10% DMF.

Sperm forward progression requires the correct functioning of several systems inside the cells to achieve motion and to overcome the hydrostatic drag [285], and depends of several factors such as ATP and cAMP concentrations and the composition of the extracellular environment [194]. Forward progression also depends on the energy storages of the cells, thus is usually time dependent [198]. Our previous studies seem to indicate that FPM is a far more sensitive motility parameter than total sperm motility and that dilution greatly reduces FPM in *A. zetek*i spermatozoa. These results suggest that FPM decreases around 60-70% compared to fresh sperm FPM and that an interaction between CPA and time, while not condition, had an overall effect on this parameter. As observed with percentage motility, our results indicate that post-exposure FPM is greater in samples equilibrated in CPA1 for 5 and 10 min, followed by CPA3. The latter seems to provide some protection to the cells for a longer period of time, since the highest post-exposure FPMs at 15 and 20 min were obtained with CPA3, although still considered poor. The interaction between CPA and exposure time suggests that the effect of CPAs in this particular parameter depends on the time the cells remain exposed to them for

equilibration. A shorter equilibration time seems to favor the recovery on higher percentages of cells with progressive motility. Besides the possible CPA toxic effects and cellular damage caused by hyper-osmotic shock, the low recovery of post-equilibration FPM might be also due to dilution at reactivation, where hypo-osmotic shock can produce swelling the flagellum and damage to the dynein-ATPase machinery, rendering the cells immotile [76]. Even though our final model for evaluation of percentage motility did not include the exposure time effect, our empirical observations indicated that exposure times of 5 and 10 min produced the best post-exposure motilities compared to 15 and 20 min, thus these two last time points were not considered for the rest of the trials. Our observations coincided with the results observed for post-equilibration FPM, where exposure for 5 and 10 min resulted in the highest FPM values. Further investigations on the effects of exposure to CPAs and post-exposure dilution on sperm motility parameters (utilizing CASA system) and membrane integrity are necessary to estimate the specific effect of these CPAs and exposure times of 5 and 10 min in *A. zeteki* percentage motility parameters during equilibration.

For the cryopreservation trials we could not increase the number of males sampled because of time constraints, nevertheless future studies contemplate increasing the sample size to reduce variability in the responses to cryopreservation of *A. zeteki* spermatozoa. The cryopreservation trial 1 (CPA1 and CPA3 alone) and trial 2 (CPA1-TYB and CPA3-TYB) resulted in no recovery of viable cells. After the equilibration study we concluded that spermatozoa exposed to CPA1 and CPA3 for 5 and 10 min at 4 °C recovered around 40% motility after reactivation, indicating that the actual freezing process may have caused the negative results observed after cryopreservation in these

trials. The first cryopreservation trial involved freezing using just one ramp 10 cm above LN$_2$, at around -60 °C, before plunging the samples in LN$_2$. This drastic drop in temperature might have not given enough time for water to exit the cells resulting in intracellular ice formation and cell death [263]. Additionally, the composition of these two CPAs might confer some protection to the cells during equilibration, while not enough for the hardships of cryopreservation. Further trials using these CPAs while slowing down the cooling rate are necessary to understand if the outcome we obtained arises from the composition of the CPAs themselves or if it is related to temperature.

The second trial contemplated changing the cooling rate previously used in trial 1, by slowing it down [adding one more ramp (-95 °C) before plunging the samples in LN$_2$] and adding additional agents to the CPAs used in trial 1. Slowing down the cooling rate and adding TEST yolk buffer to the CPAs did not improve the recovery of motile cells compared to trial 1. Kouba and Vance, 2009, reported some recovery of amphibian sperm motility after cryopreservation using TEST yolk buffer, nevertheless a negative effect was observed after dilution, where a precipitate of the buffer adhered to the cell membranes rendering them immotile. Even though this buffer has been successfully used for sperm cryopreservation in several species [209], it does not seem to confer much protection to *A. zeteki* spermatozoa during cryopreservation. The non-disclosed formula of the buffer indicates it contains gentamicin sulfate and glycerol. Our preliminary tests revealed that *A. zeteki* did not respond well to cryopreservation with glycerol, and the effects of gentamicin sulfate on the cells are unknown. It remains to be investigated if the precipitate observed by Kouba and Vance, 2009, is also affecting the recovery of motility in *A. zeteki* spermatozoa.

The cooling rate determines if the cells remain in osmotic equilibrium with CPAs and modulates intracellular ice formation [242]. Slowing down cooling rates has been reported as beneficial for sperm cryopreservation in several species, including amphibians [50,56,61,209,241,286,287]. Kumar et al., 2003, suggest that an optimal cooling rate for the cryopreservation of bull, ram and boar spermatozoa is approximately of -30 °C/min. On the other hand, super-fast cooling rates can induce cold shock [286] and cryoinjury, having a detrimental effect on amphibian spermatozoa. A study on wood frog spermatozoa showed that faster cooling rates resulted in very poor recovery of motility after cryopreservation in a CPA containing methanol [59]. Sargent and Mohun, 2005, reported that the optimal cooling rate for the cryopreservation of *X. laevis* spermatozoa is 10 °C/min. Browne et al., 1998, recovered spermatozoa from *Bufo marinus* with a high fertilizing capacity after using a slow cooling protocol at a rate of 10 °C/min. The step-wise reduction in the cooling rate we performed in the second trial might have not been the optimal for the cryopreservation of *A. zeteki* spermatozoa. For this reason we decided to include an additional ramp over the LN_2 to slow down even more our cooling rate, which was included in trial 3. More research is required on the effect of other penetrating CPAs (DMSO and glycerol) in combination with slower cooling rates to evaluate if these CPAs were indeed toxic or if it was a matter of "cooling rates" what caused no recovery of motility in this species when these CPAs were tested.

The third cryopreservation trial was the only one that resulted in some recovery of *A. zeteki* motile spermatozoa with an approximate decrease of 80-90% in percentage motility and 60% in FPM compared to fresh samples. The cryopreservation process is already known to reduce cell viability by an average of 50% compared to non-frozen

sperm samples [248], although empirical standardization of species-specific protocols has resulted in lower proportions of post-thawed cell damage [209,268]. The CPAs used in this trial had the same basic composition as CPA1 and CPA3 with the addition of 10% raw egg yolk (see preparation in the methods), 800 mM sucrose and 20 mM sodium bicarbonate. Additionally, the protocol used in this trial included slow cooling in 3 ramps above LN_2 (-55 °C, -95.7 °C and -181 °C) at 3 min/ramp. Our models suggested that condition, regardless of CPAs and exposure times, had an overall effect in *A. zeteki* post-thawed sperm percentage motility and FPM, with ARS samples showing higher recovery of motile cells than spermic urine samples. It is worth to mention that the difference on percentage motility between ARS and SU trended towards significance ($p = 0.0852$). The high variability in the individual responses to cryopreservation in trial 3, added to the need of increasing the sample size might have accounted for the fact that CPAs were not considered as a fixed effect in the final models for the analysis. The standard deviation of the residuals for the random effects was higher than the individual effect SD for both percentage motility and FPM (%Mot Individual SD: 2.49, residual SD: 8.66; FPM Individual 15.78, residual SD: 29.22) suggesting that for percentage motility and FPM there were additional random effects besides the individual variability affecting the response variables, perhaps the effect of CPAs hidden behind the small sample size. High individual variability in cryosensitivities among males of the same species has been observed in many species [288]. Individual characteristics, including genetics, contribute to males being "good or bad" freezers [209,232,289]. Part of the variability we observed in *A. zeteki* individual responses to cryopreservation might be due to these intra-specific differences. As mentioned before, motility parameters also involve the correct

functioning of different cellular systems working together and it is difficult to determine which one or if all are being affected by the cryopreservation process which might also account for the observed variability.

CPAs used in trial 3 contained sodium bicarbonate, sucrose and raw egg yolk additional to DMF and trehalose. The cryopreservative effects resulting from the interaction of the CPA components might be complex. Sodium bicarbonate, also present is ARS, is commonly used for the cryopreservation of mammalian spermatozoa and it is thought to have a stimulating effect on the motility of these cells [37,60,290,291]. Kouba et al., 2003, observed that spermatozoa from *Bufo marinus* kept in ARS showed a greater percentage of viable and morphologically normal cells than those kept in spermic urine alone, suggesting that ARS has some sort of protective effect over anuran spermatozoa. Sargent et al., 2005, observed a mild positive effect of sodium bicarbonate in the recovery of post-thawed *X. laevis* spermatozoa. Addition of sodium bicarbonate to our CPAs might have had a positive effect in the protection of *A. zeteki* spermatozoa against cryoinjury and a mild stimulatory effect on the motility parameters. The use of sucrose and egg-yolk in the cryopreservation of anuran spermatozoa has proven beneficial [57]. *Bufo marinus* spermatozoa shows improved post-thawed motility with the addition of egg-yolk to CPAs while the addition of sucrose alone improved even more the sperm motility parameters after freezing in this species [51]. Egg-yolk is thought to stabilize the sperm membrane and to replace lost components preventing further cell damage [51]. The beneficial effects of adding egg-yolk to CPAs have also been observed in several species of mammals and fish [209,236]. Addition of ARS to spermic urine increases the osmolality to iso-osmotic levels inactivating the cells. ARS might have attenuated the hyper-osmotic

shock upon addition of CPAs, having a positive effect in post-thaw motility recovery. Additionally, the effect of the three extra non-penetrating agents added to the protecting effects of trehalose and DMF, might have helped improve post-thaw motility recovery of *A. zeteki* spermatozoa.

Our results revealed that cryopreservation reduced DNA integrity by around 30-50% compared to fresh spermatozoa (see Fig 9). Our models indicated that CPAs, while not conditions or exposure times, affected *A. zeteki* sperm DNA integrity after cryopreservation, with CPA3-REY showing higher post-thaw percentage of DNA integrity than CPA1-REY. The differences between these two CPAs is that CPA3-REY contains 5%DMF while CPA1-REY contains 10% DMF. The higher percentage of intact DNA observed with CPA3-REY might be due to the lower concentration of DMF used in its preparation, indicating that higher DMF concentrations are probably detrimental to *A. zeteki* sperm DNA integrity. Higher DMF concentrations of around 12% have been successfully used for the cryopreservation of *Rana temporaria* hormonally induced spermic urine with a good recovery of motility, nevertheless the effect on DNA integrity was not assessed [61]. However, cryopreservation of tambaqui spermatozoa with 11% DMF resulted in greater DNA fragmentation than that obtained with 5% and 8% DMF [267]. DNA fragmentation is one of the detrimental consequences of the sperm cryopreservation process [248]. Human sperm DNA integrity is reduced by an average of 20% after freezing [250]. The cryopreservation process increases the production of reactive oxygen species (ROS), causing membrane and lipid peroxidation and DNA fragmentation, also reducing sperm motility [221,233]. Evaluation of DNA integrity after cryopreservation is important since utilizing spermatozoa with fragmented DNA for the application of ARTs might

directly compromise embryo survival [236,248]. Addition of antioxidants such as enzymes like catalase or superoxide dismutase or substances like tocopherol might help reduce the oxidative damage caused by cryopreservation and improve the DNA integrity of thawed spermatozoa [233]. Future studies assessing the effect of equilibration on DNA fragmentation are necessary. It is important to mention that due to time constraints, data of the effect of equilibration and cryopreservation on the morphology of *A. zeteki* spermatozoa could not be shown; nevertheless the data set is being processed for publication purposes.

The information produced by this study is essential for the development of cryopreservation protocols in the conservation breeding programs of *A. zeteki* and other *Atelopus* species, and the first step for the creation of a Genome Resource Bank for this species.

Chapter 5: <u>Research impact and significance</u>

Discussion

One common question people ask, whose answer turns out to be the main cause behind my study is: why save amphibians? The answer to this question is complex because the role of amphibians in nature and in human history and society is extensive. Amphibians are known to be environmental indicators, their physiological and morphological characteristics, such as permeable skin, a primitive immune system, dual life cycle, and being primary consumers, make them highly susceptible to changes in their environment, and therefore help us determine the health of ecosystems [18,292-295]. Amphibians play an essential role in the ecosystem because they serve as the energy transfer link between aquatic and terrestrial environments, control excess grow of algae and accumulation of organic matter in the streams, serve as primary consumers of many species of insects and small vertebrates and function as a food source for many other species [293,296,297]. The dramatic declines that have occurred in amphibian populations around the world, with entire species disappearing from niches and habitats, might already be impacting the very functionality of the ecosystems they inhabit(ed) with future, not yet seen, chain-reaction like consequences in other taxa, including humans.

The specialized skin of amphibians makes them a wonderful source of bioactive compounds and their importance in modern medicine is growing more and more [293]. Peptides, alkaloids, amines, steroids and bufogenins found in frog and toad skin have shown a wide variety of medicinal properties including cytotoxicity against bacteria, virus, fungi, protozoa and mammalian cells, anti-inflammatory, pro-inflammatory, anti-diabetic, anti-HIV, anti-cancer and analgesic activity, among others [293,298-305]. The Global

Amphibian Assessment identified at least 73 species that have some kind of medicinal value, some of which are under risk of extinction [293,306]. Protecting amphibians from disappearing might take us to great advancements in the human health field.

Amphibians also serve as an important protein source for many cultures around the world [293,307]. Subsistence harvesting by small and local communities is not too detrimental to wild amphibian populations since it is usually seasonal and sporadic [293]. Nevertheless, the growing international demand for frog legs in Asia, Western Europe and the United States has greatly impacted wild populations, mostly those from the main exporting countries such as India, Bangladesh and Indonesia [293,307,308]. Even though the "amphibian meat" trade has been estimated in more than USD 111 million for over a period of 20 years, contributing to the global economy, over-exploitation has facilitated the rapid declines and slow recovery, if any, of wild amphibian populations around the world [307,308]. The use of amphibians as a food source is one more reason that highlights the importance of this class, however, it is tightly linked to population declines in the wild by irresponsible harvesting and the pet trade.

The facts that I have mentioned above are some of the reasons why we should save amphibians, an immensely diverse, important and beautiful group. For highly endangered species, only found in captivity, the development of captive breeding programs might be the only chance for survival. Understanding the reproductive biology of these species is usually a complicated task, accomplished by studying one species at the time. The Panamanian golden frog is one of the most charismatic amphibian species of the world and it is classified as critically endangered by the IUCN (2014) [20,95,309]. Experts even believe that the species might be extinct in the wild since no sighting has been recorded

for at least the past five years [25,94,310]. A closely related species, *Atelopus varius*, is still found in the wild but their populations are rapidly declining [22,94,311].

The first conservation efforts for the species started in 1999 with the creation of Project Golden Frog, when several Panamanian and US institutions came together to elaborate an action plan to save the species from extinction due to the imminent threats of chytridiomycosis, habitat destruction and pet trade [22,23,93,94]. Fifty individuals (19.19.12) were extracted from the wild and, given the limited holding space and resources available in Panama, CITES exportation/importation permits were issued for the relocation of the animals to AZA institutions in the United States in the year 2000, mainly in the Maryland Zoo (former Baltimore Zoo) with the implementation of a studbook and limiting their distribution to AZA-accredited institutions only [20,22,23,94]. El Valle Amphibian Conservation Center (EVACC), located in El Valle de Antón, Panama, opened its doors in 2007, with support from the Houston Zoo, as a continuation of the amphibian conservation initiative for the formation of assurance colonies of Panamanian threatened amphibians, including the endemic *A. zeteki* [20,22,94]. In 2009 the Panama Amphibian Rescue and Conservation Project was formed with the collaboration of many more institutions from Panama and the US, with its subsequent relocation to Gamboa (now the Gamboa Amphibian Rescue Center) and the construction of state of the art facilities for conservation and research of endangered amphibians in Panama [94].

Due to the limited knowledge available and the urgent and rapid establishment of these first formal captive colonies given the extinction risk the species faced in the wild, researchers and institutions have been slowly learning about *A. zeteki* ecology, physiology and reproduction in order to maintain healthy captive populations. The

Maryland Zoo has successfully accomplished breeding in the species for some time now while EVACC has started recently with some success [94]. The Panamanian Golden Frog (*A. zeteki*) husbandry manual from the National Aquarium in Baltimore provides detailed information and recommendations for the successful maintenance of a captive population from this species [312]. The manual points out several important topics for that captive breeding for *A. zeteki*: reproduction is not as easily accomplished as in other anuran species in captivity, dystocia is a common cause of female death, long-term breeding colonies need to maintain 30 to 50 animals, breeding pairs should be kept in tanks no smaller than 15 gallons, excessive production of offspring requires culling to minimize space problems and to keep healthy population numbers, some amplexant pairs will not successfully breed and it might take up to one month for the females to spawn the eggs. Besides this information, the Manual also advises the quarantine of animals transferred between institutions or brought from the wild, with specific shipping recommendations.

The implementation of Assisted Reproductive Technologies (ARTs) in *A. zeteki* captive breeding programs might attenuate some of the above-mentioned circumstances and help improve the efficacy of such programs, not to mention facilitate the start of new programs, like the Gamboa ARC in Panama. The work presented in this doctoral dissertation has provided new and important information on the reproductive biology of the male *Atelopus zeteki* and the sperm cellular biology of the species. This information will help in the implementation and improvement of existent and future captive breeding programs of this and other related critically endangered species. We now have new, otherwise missing information on the ejaculate parameters of this species obtained after hormonal stimulation, including average spermic urine volume, osmolality, pH and

sperm concentration. The general morphology of *A. zeteki* spermatozoa and the average sperm DNA integrity has also been described. Additionally, the species response to hormonal stimulation and its influence in the parameters mentioned above is critical information needed for the implementation of ARTs.

The use of hormonal stimulation in captive programs has been implemented to stimulate reproductive behaviors, overcome asynchronous breeding episodes, stimulate spawning in otherwise egg-retaining females and for the particular collection of gametes to be used in the implementation of specific techniques such as cryopreservation, artificial fertilization (AF) or intracytoplasmic sperm injection (ICSI) [11,32,33,62,64,65,130]. Our work showed that males *A. zeteki* respond well to stimulation with a variety of hormones and concentrations producing high quality sperm samples, contrary to what is observed in other species that are highly selective on their responses to specific hormone treatments [145]. Different hormones and doses stimulate the production of more or less concentrated samples and the criteria for the selection of a treatment should be based on the researcher's immediate needs. For example, the recommended sperm concentration for the application of AF should range between 10^5 and 10^7 cells/ml [32,60]. In our study, all hormonal treatments yielded an average of 10^6 cells/ml, suggesting all of them stimulate the production of enough spermatozoa for its use in AF. While Browne and Zippel, 2007, suggest that sperm concentrations obtained in spermic urine are usually less than 10^6 cells/ml, our data shows that is not the case in male *A. zeteki* when stimulated with GnRH, hCG and Amphiplex (see concentrations in chapter 1). For cryopreservation purposes, where the freezing/thawing process reduces the viability of the samples in an average of 50% [232,235] and higher sperm concentrations are needed, we recommend the

use of Amphiplex since it stimulates the production of higher concentrated sperm samples in *A. zeteki* than the rest of the treatments and concentrations, thus increasing the probability of recovering a higher percentage of viable cells. The successful application of hormone treatments for the collection of viable spermatozoa from this species suggests that similar results could be obtained from related endangered *Atelopus* species such as *A. limosus*, *A. certus* and *A. glyphus*, currently held in captivity in the Panama Amphibian Rescue and Conservation Center. Successful application of such protocols for captive breeding programs could help us keep and reproduce valuable specimens from these three species in captivity that have been shown to contain important bioactive toxins in their skin with possible medicinal use [313], until reintroduction is a viable option.

The motility parameters obtained in our study, all higher than 50%, indicate that these hormone treatments are not detrimental for *A. zeteki* sperm quality. Motility is the most commonly used parameter to estimate sperm quality and fertilization potential [167,194,207], nevertheless, other not so evident but equally important parameters have been recently included in the assessment of sperm quality. Evaluation of the sperm DNA integrity suggests there is an average of 40% of the cells with fragmented DNA in hormonally stimulated and testicular *A. zeteki* spermatozoa. Further research is necessary to learn what the "normal" (threshold) percentage of sperm DNA fragmentation is in the species and the effect of what it seems a high percentage of damaged DNA, compared to that in humans [137,314], in the sperm quality [84,133] and if it accounts for viable cells with fertilizing capacity [173]. This information is particularly important for the specific application of ICSI, where spermatozoa with damaged DNA could be ingenuously selected [133,184]. Fertilization tests using hormonally stimulated spermatozoa, with a parallel assessment

on DNA fragmentation, could shed some light on the effects of such percentages on the fertilization capacity of the cells, their association with motility parameters and its impact in embryo development [315]. Some studies have recently pointed out the importance of the sperm DNA integrity assessments and that such tests might be better predictors of the fertilization success than regular sperm quality parameters like motility and forward progressive motility [315]. The application of other reliable tests, such as Comet Assay, could be useful to confirm the results obtained with TUNEL assay in the evaluation of DNA integrity of *Atelopus zeteki* spermatozoa and to increase the battery of tests available for the species [177,178,181].

The general morphology of *A. zeteki* spermatozoa is similar to that described in other species within the Bufonidae family [55,81,171,172]. Future ultrastructural assessments are necessary to better understand the physiological responses of these cells to changing extra and intracellular conditions. Our data revealed the presence of the mitochondrial vesicle (MV) in *A. zeteki* spermatozoa. This structure has also been described in other species of Bufonidae and seems to be related to sperm motility parameters [55,81]. Other ultrastructural and physiological studies are necessary to better understand the role of this structure in *A. zeteki* sperm functionality, that provide more information on the location, quantity and metabolic activity of the mitochondria found inside the MV and of those distributed along the cell body (head, midpiece and tail) and their relationship with the motility parameters and fertilization capacity in the species. This information will also be important to understand how this structure might be related to changes in metabolic activity when the cells are exposed to different extracellular environments (osmolality and temperature), its relationship with sperm motility velocity and duration, cell viability

and survival before and after cryopreservation. This information altogether will help develop better handling and ART protocols for this and other related species.

Even though PGFs are believed to be seasonal breeders in the wild [29] like many other anurans that inhabit tropical regions with marked dry/wet seasons [316], such natural cycles can be bypassed with the use of hormonal stimulation [62]. Our results show that males *A. zeteki* are capable of producing spermatozoa with similar average quality year round when hormonally stimulated. Seasonal breeding species face great challenges in captive scenarios where the necessary environmental cues are missing or altered [145], affecting natural hormonal cycling, gonadal maturation and finally altering breeding episodes. Our results show that *A. zeteki* breeding programs could implement the use of hormone treatments to stimulate the production of spermatozoa out of the known breeding season with no decline in sperm quality parameters. In the wild, males *A. zeteki* remain close to the streams year round while the females move deeper into the rain forest during the wet season and come back to the streams at the beginning of the dry season to breed [23]. There is no information available on the species seasonality in captivity. Future studies should evaluate if males *A. zeteki* are truly seasonal breeders or if they are capable of producing spermatozoa throughout the year, depending only on female availability, assessing the effect of captivity in this reproductive behavior. This information could be highly useful for the correct application of ARTs in captive populations of this and other related species.

The positive response of males to the different hormone treatments we used in this study suggests that females *A. zeteki* could respond similarly to these treatments as well. Future studies evaluating the effect of hormonal stimulation on *A. zeteki* ovulation and

spawning should include treatments with Amphiplex, GnRH and hCG, trying out different concentrations, assessing if the use of priming doses prior to higher ovulatory doses are necessary, evaluating their success at stimulating spawning. Hormonal priming have been successfully used in females from other Bufonid species like *Bufo baxteri* where a single dose of a GnRH/hCG cocktail fails to induce spawning, requiring the administration of one or two lower doses up to a week before of administering a higher final ovulatory dose [62]. As the husbandry manual for the species points out, egg retention is a very common cause of death in females *A. zeteki* [23]. The use of hormonal treatments might help reduce the number of genetically valuable females lost by this condition [11,65,130]. The implementation of appropriate hormonal stimulation protocols for the production of gametes in *A. zeteki* captive breeding programs can specifically tackle the lack of breeding behavior, asynchronous breeding episodes, non-spawning amplexant pairs and dystocia.

The implementation of ARTs requires the correct handling of gametes to preserve their viability and fertilization potential for the longest time possible [207,209]. Chapter 3 aimed to understand the effects of extracellular conditions (dilution and temperature) in the motility parameters, morphology and DNA integrity of *A. zeteki* spermatozoa, to further apply this information in the development of sperm handling protocols. Short-term storage (in temperatures above 0 °C) of gametes is necessary when collecting sperm in the wild or in captivity for transportation purposes, when synchronizing breeding pairs through the use of hormones in the application of AF or ICSI or for the preparation of samples prior to cryopreservation [317]. Spermic urine samples contain already motile spermatozoa since activation occurs after contact with lower osmolalities [11,76]. Long

exposure to hypo-osmotic environments negatively impacts amphibian sperm viability [76]. Our results showed that long-term dilution in water is clearly detrimental to *A. zeteki* sperm motility parameters (mainly FPM), DNA integrity and sperm morphology. Such effects can be mitigated if samples are kept in spermic urine at 4 °C, where motility and FPM remain higher than 50% for up to 46 minutes after collection; DNA integrity and intact morphology also remain higher than 50% and 40% for up to 23 min after collection respectively. After assessing these results we now know what are the best conditions and times to keep hormonally stimulated *A. zeteki* spermatozoa viable after ejaculation. Additional studies estimating the osmotic tolerance of *A. zeteki* sperm plasma membrane, critical osmolality and hydraulic conductivity (Lp) would help us understand the effect of dilution and time in cell volume regulation before critical damage occurs [40,42,44,233]. This information is highly valuable for the future implementation of ARTs in the captive breeding programs for this species. Future studies evaluating the molecular pathways involved in the activation of sperm motility in this species could help us better understand the effect of osmolality in the motility parameters, the metabolic and energetic needs of the cells associated with duration of motility and viability, their association to post-thaw recovery of motile cells and finally, to *A. zeteki* sperm longevity and fertilization capacity. Including the use of Computer-Assisted Sperm Analysis (CASA System) in the future assessment of motility and other related parameters, such as sperm velocity, is essential to gain a better knowledge of the effects of extracellular conditions in *A. zeteki* spermatozoa. The evaluation of the effect of long exposures to low temperatures in the fertilization potential of the cells is also necessary [272].

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Successful cryopreservation protocols are essential for the creation of Genome Resource Banks, especially for highly endangered species. My work on *A. zeteki* sperm cryopreservation is a first step aimed towards the creation of an amphibian GRB at the Smithsonian National Zoological Park and the Panama Amphibian Rescue and Conservation Center, for the preservation of extremely valuable genetic diversity that might be lost in the coming years. For the development of adequate sperm cryopreservation protocols is necessary to understand the pre and post freezing effects of CPAs and exposure to anisosmolar conditions [209,236,253]. Our results showed that equilibration for 5 and 10 min at 4 °C with CPAs containing 10% trehalose in combination with DMF in spermic urine samples or in samples pre-diluted in ARS showed some recovery of *A. zeteki* sperm motility parameters. For the actual freezing of *A. zeteki* spermatozoa, addition of 10% egg-yolk, 800 mM sucrose and 20 mM sodium bicarbonate to the existent CPAs resulted in recovery of around 20-30% of motile cells. Step-wise cooling, initiating with equilibration at 4 °C for 5 min followed by exposure to LN_2 vapor at -55 °C, -95.7 °C and -181 °C (3 min/ramp) seemed to improve the results observed in the previous unsuccessful trials. We recommend inactivating the samples pre-freezing in ARS since this solution provided higher recovery rates in percentage motility, FPM and DNA integrity. CPA3-REY resulted in higher recovery of cells with intact DNA, therefore we think this is a better candidate to continue our cryopreservation trials.

The results from this body of research will have meaningful and important applications and a positive impact in the improvement of captive breeding programs for this species and for the amphibian conservation world. Now, the Panamanian Golden

Frog can serve as a model for the study of other critically endangered *Atelopus sp.* (i.e. *Atelopus limosus*, lowland and highland) being held around the world, especially in EVACC, Gamboa ARC in Panama, or in US institutions to initiate or to improve captive breeding programs for the conservation of endangered species of amphibians.

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