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Cryopreservation of spermatozoa in different species of chameleons

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Table of Contents

Abbreviations list and appendices:

Abbreviation:	Full text:
CSG	Chameleon Specialist Group
ART	Assisted Reproduction Technologies
AI	Artificial Insemination
CF	Conventional Freezing
URF	Ultra-Rapid Freezing
CPA	Cryoprotective Agents
PBS	Phosphate buffered saline
AUV	Ad usum veterinarium
mg	Milligram(s)
µg	Microgram(s)
Kg	Kilogram(s)
ml	Milliliter(s)
µl	Microliter
®	Trademark
Ca	Calcium
Mg	Magnesium
h	Hour(s)
s	Seconds
mA	Milliampere
V	Volts
SSTs	Sperm Storage Tubules
EEJ	Electroejaculation

Tables

Figures

Introduction:

Chameleons (family: Chamaeleonidae) are part of a distinctive and highly specialized family of lizards. They differ from other lizards by showing specialized abilities for which they are known, these include aptitudes like color change, zygodactylous feet, and most famously their elongated, projectable tongues. Some of them still being researched as we speak and being topics in constant evolution, for instance we now know that their ability to change color is primarily used for communication and thermoregulation rather than camouflage. This family, which is part of the order Squamata and includes approximately 200 species distributed primarily in continental Africa and Madagascar, with a few species in southern Europe, India, and the Middle East. Chameleons exhibit a wide range of morphological adaptations to survive in the forest and sustain their airboat lifestyle, such as independently mobile eyes and prehensile tails. Fossil evidence suggests that chameleons originated during the Paleocene epoch, around 65 million years ago, indicating a long evolutionary history that has resulted in the diverse forms observed today.

Today, chameleons are among the most popular reptiles, with a growing demand for them arriving on the market every year. However, as the demand for these new types of pet emerge, new challenges are brought with them, both on medical and economical levels.

Due to human activities, both directly and indirectly, a vast amount, if not most, animal species are facing existential threats, making the survival of their kind more and more difficult. Unfortunately, this situation has not evaded the chameleons. Indeed, at least 33% of chameleons are considered by the Chameleon Specialist Group (CSG) threatened, of the 200 currently recognized species of chameleon, 66 are assessed as threatened (i.e., Critically Endangered, Endangered, or Vulnerable) and an additional 35 are assessed as Near Threatened. Additionally, nine species of chameleon are considered Critically Endangered! These species are considered to be facing an extremely high risk of extinction in the wild and are in immediate need of conservation action.

Animal conservation englobes a variety of strategies, including ex-situ methods such as zoological parks and gene banking. Humans have devised numerous techniques to study fauna and safeguard species from extinction. Recent advancements have introduced innovative approaches to increase the conservation efforts, emphasizing the importance of understanding and mastering the reproductive physiology and biology of species to avert their extinction. Despite extensive research on the reproduction of other reptile and lizard species,

the chameleon genera remain understudied and unaccounted for, leaving too many gaps needing to be filled. The state of research regarding cryopreservation of reptilian semen, is still a vastly unknown topic that needs more focus allocated to it. This study aims to evaluate a set of cryopreservation protocols using a different preservation media, we hope that through this, a better understanding can be made to further the efforts of scientists in squamata and more precisely chameleon conservation.

Through this thesis research, our aim to provide the first pilot study on cryopreservation of sperm of chameleons species, namely the veiled chameleon (*Chamaeleo calyptratus*), the Carpet Chameleon (*Furcifer lateralis*) and lastly the panther chameleon (*Furcifer pardalis*).

Our goal is to explore the feasibility of using semen cryopreservation as a tool to conduct effective reproductive campaigns for these reptiles. In the future, it can potentially aid in the conservation of these species and improve captive breeding programs.

We will go through the whole process, beginning with retrieving spermatozoa samples buy mean of postmortem dissection before preparing the samples for cryopreservation with different semen extenders and cryopreservant agents, comparing which provided the best outcome when subsequently thawing them before performing detailed analyses on the sperm, evaluating both survival rates and motility to assess the success of the cryopreservation process.

Our aim is not only to retrieve the highest possible number of viable sperm cells post-thaw but also to maintain robust motility, which is crucial for fertility. Achieving good post-thaw motility would demonstrate the potential of cryopreservation for use in reproductive technologies for reptiles, enabling future applications in genetic management, population diversity, and conservation efforts, particularly for endangered or at-risk species. Additionally, this research will contribute valuable data to the relatively unexplored field of reptilian cryobiology, setting a foundation for further studies in the cryopreservation of other reptile species.

Literature review

1. Chameleon's biology

1.1 Taxonomy

Throughout the world, the word "lizard" is often used to as an umbrella term to describe reptilian animals with the ability to walk using their legs rather than serpentine like a snake would do. However, just like for the rest of the animal kingdom, lizards are harboring a far more complex taxonomy than most would think. Indeed, all reptiles fall under the class "Reptilia", itself under the phylum "Chordata", the taxonomy then declines itself into similar looking yet very different orders: "Crocodilia", "Squamata", "Testudines" and lastly "Rhynchocephalia" [2].

The word "chameleon" in itself could be considered as an umbrella term as the number of species is large and, like most experts agree, greatly underestimated. Indeed, while more than 200 species and 13 subspecies have been described an unknown, yet not negligible number of species were not yet discovered nor described. Approximately 55% of these species originate from continental Africa, 44% from Madagascar, and the remainder from Europe the Middle East, Asia and other offshore African islands. From these 200 species, we can draw up, thirty distinct genera and subgenera that have been described, with Gray (1865) contributing 14 genera in a single publication [2]. Of these genera, 22 were described before the end of the 18th century, while only 8 have been described since 1900 [1].

1.2 Reproductive anatomy:

Chameleons are reptilians, as such they share similarities between each other, especially in the so-called cloacal area, indeed, reptiles do not have separate external openings for the waste products of the urinary and digestive tract [33].

Instead, the cloaca is divided into three regions: the most anterior section, called the coprodeum, receives waste from the large intestine, a bit like the mammalian anus; the middle region, known as the urodeum, receives the ureters, and genital ducts. In some species, the genital ducts and ureters enter the urodeum separately, while in others, they fuse before entry; the final and most caudal section, the proctodeum, collects waste and urogenital products, which are then expelled through the vent, into the environment [33]. The proctodaeum in chameleons is keratinized along its entire length, but is relatively short compared to other taxa [34].

The anatomy of the of the chameleon, just like most other vertebrates and member of the animal kingdom in general, may be divided into male versus female reproductive anatomy [34].

In female, the gonads, namely the oviduct follicles and eggs or embryos, take up a rather large position of the body cavity, whereas in the male, the testis do not take as much space. The testicles are black and the seminal vesicles production seminal fluid, are arranged in a tubular way [1]. Testes in the chameleon are similar to the other lizards and other squamates in the fact that they are located intra-abdominal and are closely related cranial to the kidneys since their formation during the embryonic development stage. They are round or slightly oval and it is interesting to note that although internal, the testis of male chameleons are in fact pigmented and not just plain black. The testicles themselves are held together inside the tunica albuginea, a thin fibrous tissue, because of its very fine nature (3–15 μm), it allows you to visualize the coiled seminiferous tubules just under this exterior connective tissue layer; this also makes the testis very soft upon digital palpation [34]. When sperm is being produced by the testicles, they can easily double in size, this can produce high intra-testicular pressure which has for effect to give the testis additional firmness as well as giving the rest of the internal organs less space to develop [34].

The epididymis lies adjacent to the testis, appearing as a straight opaque tube in immature individuals, but becomes coiled in mature ones. During the active season, it swells as it fills with sperm. The epididymis continues into the ductus deferens, which straightens as it extends toward the back and runs ventrally beneath the kidney. The ductus deferens merges with the ureter behind the kidney and in front of the cloaca, forming a ductus deferens-ureter complex. This structure enters the cloaca dorsally via the urogenital papilla, this may differ from species to species. [34]

The special characteristic of male chameleons (and other member of the squamates) lies in their penile anatomy compared to other members of the animal kingdom. Indeed, they posses what is called a “hemipenis”, in fact, they have pair hemipenises. They are kept inside of the body and lie in an inverted position at rest, back into the cloaca of the animal [1].

The hemipenises are located in a pocket at the base of the tail, which might then form a noticeable bulge, this can be useful when trying to determine the sex of the individual. In chameleons, the shape of the hemipenis can vary significantly from one particular specie to

another. The shape may have a strong or weak clavate shape, or it can be sub-cylindrical when everted [1].

Anatomically speaking, the hemipenis itself is composed of three main parts: the pedicle, the truncus, and the apex. The pedicle is the part most proximal to the rest of the body of the animal and it has a relatively smooth surface. The truncus, which is the medial portion, can either be composed of calycules who features reticulated pits, the “calyces”, or be acalyculate, with a smooth surface. This can make the differentiating the pedicle from the truncus challenging [1].

During copulation, sperm is transported by a channel-shaped groove which is called the sulcus spermaticus. It runs along the hemipenal surface of the pedicle and truncus, bordered by sulcal lips. The sulcus spermaticus itself is smooth, while the sulcal lips can either be smooth or have ridge traces from surrounding calyces. These sulcal lips, just like the truncus previously mentioned, may also exhibit a capitate state. There, they diverge distally to go on to form a clear ridge boundary between the truncus and apex, they can also be noncapitate. The apex of the hemipenis is typically simple or slightly having two lobes at its distal end and is often a very sophisticatedly ornamented. This sort of embellishment is usually arranged bilaterally and can include a variety of structures such as horns, crests, pedunculi, papillae, auriculae and rotulae [1].

1.3 Reproductive physiology:

Reproductive studies regarding chameleons as a whole is a greatly under-researched ordeal, which unfortunately leaves us with little knowledge on the matter. However, what we do know some interesting details on their reproductive cycle and how, as a group, their “cycle of life” works. Chameleons as a group is composed of different species, which, just like in any other animal order, may be significant and impact research, but they can also be regarded as the most similar to each other rather than comparing results to other reptilian species (although those might also give us clues on how to progress) [32].

In studies, we learn that veiled chameleons (*Chamaeleo calyptratus*) follow a prenuptial reproductive strategy, whereas panther chameleons (*Furcifer pardalis*) exhibit a postnuptial reproductive approach. Furthermore, research showed that chameleons can safely undergo repeated electroejaculation under anesthesia twice a month for an entire year, without significant mortality or the development of clinical disease related to the procedure [32].

An interesting and important fact about the chameleon's reproduction is that they are able to undergo embryonic diapause [34]. This method of reproduction allows the chameleon female after the copulation with the male, to temporarily stop the development of the embryo and keep it in a seed-like form in the uterus although poor reproductive conditions might exists, so that the development of the embryo may be resumed when better circumstances are met for the female [34]. This development suspension of the embryo is an amazing reproductive strategy that has been frequently used across the animal kingdom [35].

It is estimated that more than 130 species of mammals use this method of reproduction [35], including marsupials, rodents, mustelid, ursids and phocids carnivores, roe deer, bats and armadillos [36].

In chameleons, embryonic diapause not only allows for delays development of the embryo itself but also delayed hatching to maximize seasonal environmental settings and increase offspring survival [34].

Female chameleons not only are able to use embryonic diapause, but they are also able store sperm within their bodies after copulation without subsequent fertilization. They may then keep the sperm cells alive, and the fertilization process may be carried out later on. This is possible because of Sperm Storage Tubules, also called "SSTs" [34].

These tubules are located in the non-glandular uterus of female Chameleonidae and was first described in 1962 by French scientist St. Girons [37]. He explains that Successive fertilizations, by a batch of remaining sperm stored for several estrous cycles, are possible although very rare, and that this phenomenon has been observed in breeding salamanders, turtles, tropical snakes, and what is most important to us: Chameleons [37]. He noted the presence in all individuals, at the distal end of the oviduct, of seminal receptacles containing spermatozoa, while there was an absence of the false tubular glands on the the proboscis [37].

These storage tubules were not only present in chameleons, but also in other reptilian species, 27 within 8 families to be exact. Indeed, species like the rock agama (*Psammophilus dorsalis*), the collared lizard (*Crotaphytus collaris*) or the side-blotched lizard (*Uta squamata*) also display similar capabilities although the SSTs in their respective cases are located in different parts of their anatomy [34].

Reproductive investment, which is the energy, resources, and time an organism invests in reproduction, including activities like gamete production, mating, and parental care, is a key

parameter to take into consideration when talking about reproductive physiology of an organism. It can be divided into the number of spawns, spawn size and the size of the eggs or offsprings by the average female of that particular species. Each of these parameters may vary depending phylogenetically and geographically. [34].

In the reptilian world, this can take many different forms as species like the green anole (*Anolis carolinensis*) will produce a single egg at a time but will reproduce frequently and have a long reproductive season, thus being considered as high reproductive investment. In contrast, snow skinks (*Carinascincus microlepidotus*), which originate from Tasmania, reproduces at a low rate with some having a 14 months gestation, retaining embryos over winter and giving birth to one or up to five offsprings the following spring [34]. The female chameleon will reproduce more frequently despite the fact that they lay tens of eggs in a single clutch [34].

The end result of reproduction being offspring production, animal may give birth to either fully formed youngs which may then go and live their life independently, this is called viviparous; the other alternative is oviparous where females lay eggs in which the fetus will develop into outside of the body [34].

In chameleons, most of the species are oviparous but some have been shown to produce live offspring and be viviparous like the *Trioceros* chameleons, a genus of chameleonidae native from Africa that gives birth to 8 to 30 live born babies [34].

2 Collection methods

2.1 Cloacal massage

Cloacal massage is a technic that consists of gently, yet firmly, applying pressure onto the cloacal area of the animal, thus mimicking sexual intercourse and triggering the ejaculation in order to get a fresh, unaltered sperm sample. Cloacal massage has been described in a wide variety of species including crocodilians like the Spectacled Caimans (*Caiman crocodilus*) [7] or the Australian saltwater crocodile (*Crocodylus porosus*) [6], lizards like the Crevice swift (*Sceloporus torquatus*) and the Mexican plateau horned lizards (*Phrynosoma orbiculare*) [3] or even snakes like the Brazilian Rattlesnakes (*Crotalus durissus terrificus)* [5].

The technic, similar in all cases still has species specific differences, caused by different factors, like anatomy for instance, as even if their anatomy are similar, there are still

variation, like sizes of these animals. Seasonality may also be a decisive component as different species have different reproductive periods. These differences in technics of sperm collection via cloacal massage but also their common points will become more evident as I describe them further in the different species it has been described [5].

Technic for cloacal massage of crocodilian has differences with other reptiles, first of all, since it is not part of the order squamata, it doesn't have a hemipenis but a phallus, that is anatomically more similar to mammalian penises than that of squamates. This means, that for the massage, you first need to expose the phallus from the proctodeum (part of the cloaca) by placing a gloved finger in the cloaca and carefully pulling it outside, after that, a finger is to be placed behind the base of the phallus in the cloaca, before massaging in a proximal-distal direction the final portion of the vas deferens from beginning in the base of the phallus [7].

You can either sedate them to ensure safety of the animal and the staff, this is the safest and best technic in case of a large individual like a saltwater crocodile [6], but it can also be done by placing them on dorsal recumbency, as this position is known to calm down crocodiles [7].

For us, lizards are more interesting to look at when you talk about cloacal massage in hope of adapting it to chameleons. Indeed, not only do they have a cloaca as well (like the crocodiles previously seen), but they also possess hemipenises (unlike said crocodiles). Technic is similar the one previously seen in the preparation phase, although, they have to be sedated since placing them on their back does not cause them to relax. Anaesthesia with pentobarbital sodic intraperitoneally has been proven to work and be effective for that effect (1,6mg/ 1000g body weight) [3].

Immediately after the said sedation, the animal was placed in a dorsal decubitus position, a manual distention of cloaca was then performed, and pressure was applied at the same time to the genital papilla to obtain semen. Volumes between 2.7 and 8.5 µl are shown be possibly collected in the Crevice Swift (*Sceloporus torquatus*)[3].

In snakes, the anatomy and the method appear to be quite similar to the one seen above in lizards. Indeed, after the removal of dirt and cleaning of the cloaca and it's surrounding area, the cloacal itself was lavaged with physiologic saline. The sperm was collected by ways of gentle cranial-caudal digital massage of the caudoventral third of the snake, the semen itself that was expelled from the vent was collected with a tuberculin syringe [8]. With this method, volumes obtained may be very variable with 18.527 +/-5.61 µl in the Brazilian rattlesnakes *(Crotalus durissus terrificus*) [5].

2.2 Electro-stimulation ejaculation

Electro-stimulation is a procedure where you produce an electrical current with a small probe in order to produce an ejaculate from the animal. It is widely used in zoo medicine but also in veterinary medicine in general as it is an alternative to penile hand massage, which requires labor and training, often difficult and which in the zoological field can be challenging do to the possible danger it represents for both the patient and the staff [9]. This technic has also be seen human and has been hinted as a possible therapy for early ejaculatory syndrome [10].

The technic itself varies little between animal species as the overall technic stays the same. The procedure itself can however be done in various ways. Indeed, the voltage and current themself applied via the probe may differ as the technic is more and more fined tuned. Traditionally, we apply relatively high voltage of 3–15 volts as well as a current of 900 mA maximum, nowadays new studies suggest that because of resulting high level of stress and discomfort, it may need revision and to be adapted [9]. In October 2020, a study from Yu-Hsin C. et al., showed that "low-voltage electro-ejaculation procedure using 2–3 volts with a maximum current of 500 mA can efficiently stimulated ejaculations in zoo captive lanyu miniature pigs with a high success rate of 81.3%". This is important as it shows that not only can a functional ejaculate can be obtain, but that with this new parameter, we can lower further stress thus making the sample of better quality [9].

In reptiles, electro-ejaculation has also been used to recover male gametes to be used for reproductive purposes. Stress playing a big role in the safety of reptilian patient, cloacal massage can sometime be difficult to render stress free or even low stress, electro-stimulation can then be seen as an alternative to decrease the length of time of the procedure and so decreasing the overall stress of the animal.

It is usually done under sedation, once again to try to decrease the stress during the procedure. In the crevice swift (*Sceloporus torquatus*), anesthesia can be performed using sodium pentobarbital intraperitoneally [11], you may also use protocol in Green iguanas [12]. It is worth to note that studies have shown that in chameleon species anesthesia can safely be applied twice a month during the whole year, without adverse effect like excessive mortality or the unfolding of procedure related clinical disease [32].

Once the sedation is performed and effective, the body of the animal may be cleansed using a 3% sodium hypochlorite solution and also the cloacal area should be cleaned with saline solution and any dirt shall be removed to ensure a clean and quality sperm sample [11].

After that, studies in small reptiles have shown that for the next phase of the procedure, you may start with the delivery of the discharges, these can be given the following parameters: 2.0 volts at 150 hertz of 0.75 mA. Each of the discharges should last no more than 3 seconds with intervals of 2s of relaxation in between with a maximum of 5 consecutive discharges, before allowing a rest period of 5s before restarting the cycle [11].

In green iguanas, the voltage may be increased as the sizes of the animals are much bigger than that of the small lizard that I talked about previously in the paragraph above. Indeed, electroejaculation was achieved on the animals by performance of 15 cloacal intromissions at 4 volts (V) followed by 15 cloacal intromissions at 6 V. However, unlike the protocol above the respect period in between each shock is much bigger, a period of 3 minutes was allowed between each series of electroejaculations. The green iguanas can shocked up to 3 times if this protocol was to be followed [12].

In terms of volumes, the electro-ejaculation of the Crevice swift (*Sceloporus torquatus*) gave a volume ranging from 2.0 to 9.0 µl, with an average of 4.6 ± 3.1 µl [11], while the Green iguana (*Iguana iguana*) gave a median volume on average of 0.05 mL (0.01 to 0.37 mL) [12].

It is useful to note that electro-ejaculation can not only be performed on lizards but may and was in fact used in other reptiles. For instance, that technic was also used in members of the order Testudines species like the Olive Ridley Turtle (*Lepidochelys olivacea*) and the Hawksbill Turtle (*Eretmochelys imbricata*) [14].

Electroejaculation has been attempted in chameleons, with a study that attempted the procedure on veiled chameleons over a 1-month period. During this study, only transient cloacal mucosa inflammation after each EEJ event was noticed with no chronic changes [33].

2.3 Postmortem dissection

Unlike the previous two technic mentioned, namely “cloacal massage” and “electro-ejaculation”, this technic, involving the removal and subsequent dissection of the testis and the epididymis, must be performed on an animal following euthanasia, castration or directly after death.

This technic has been documented in species like the Yellow spotted monitor (*Varanus panoptes*), a species of lizard originating from northern and western regions of Australia and southern New Guinea [15], as well as in the Spectacled Caimans (*Caiman crocodilus*) [7].

This technic aims at maximizing the number of gametes obtained, the amount of sperm in milliliters can be quite variable which may decrease the overall number of sperm and so, make further analysis and studies harder [34].

Post-mortem dissection of the reproductive tracts in the Yellow spotted monitor describes the technic as followed: “Immediately post-euthanasia, each animal was carefully dissected for the removal of both reproductive tracts. The testes and male genital ducts of lizards are intra-abdominal and paired with both tracts contributing to the production and storage of spermatozoa (left and right testes are of similar size) [34]. It is important to take out not only the testis but the epididymis as well as the vas deferens. Indeed, the testes are where the gametes are produced and are then stored inside the epididymis, similarly to mammals and other animals’ reproductive biology, but in reptiles the vas deferens is also considered as a very important place for the storage of sperm cells and so should also be taken out [15].

Postmortem dissection just like the name implies it, should be performed directly after the death of the animal or at least within a few hours. While some may argue that sperm can be viable inside an animal's body for a prolonged period of time after death with values ranging from a few hours up to a day a half depending on the species, the decrease in quality is difficult to predict and measure [16].

In case of euthanasia, welfare of the animal should be taken into account and strict guidelines must be followed. To do so it is important to place in animal in a state of general anesthesia. For induction of such state, different techniques are possible, namely using injectable anesthetic with propofol or alfaxalone. Other injectable drugs may be used, for instance, combination of ketamine, midazolam, a-2-agents, alfaxalone and opioid agents may-induce required depth of anesthesia [33].

Induction of general anesthesia may also be done using inhalation of gas agents, indeed isoflurane can be routinely used to induce sleep, although high dose of the agent must be applied and timing of the procedure may be variable [33].

For the euthanasia of the animal, it can be done in various ways like using an injection of pentobarbital intravenously or intra-cardiac. This is the more “regular way” of inducing euthanasia in small reptiles, especially intra-cardiac as the veins are sometimes hard to puncture. This technic was used on Spectacled Caimans (*Caiman crocodilus*) and it required the induction of an overdose with propofol and potassium chloride 15%. It ensures a stress free and painless euthanasia for the animal [7].

We can also use another method consisting of performing a blunt cranial displacement with the immediate severing of the spinal cord right after it. This ensures that no anesthetic or euthanasia drug will affect the viability of the sperm [15].

3 Cryopreservation of male gametes

Cryopreservation of semen is a widely used practice around the world in many mammalian species. Indeed, wether it is for conservation efforts in endangered species or in case of farm animal in case of food production, sperm is routinely collected, cryopreserved and in some cases even inseminated in a wide range of species, from dogs to cows through pigs and horses [18,19].

Motives for such practices is remarkably diverse. In farm animals for example like in cattle, it allows us to save a considerable amount of time as we can inseminated multiple cows with one ejaculate, live animal shipment may also decrease thanks to it as well as sexing the offspring [18]. The Food and Agriculture Organization tells us that: “The average sperm production of a bull ranges between 3.6 and 12 x 10^9 sperm per ejaculate. When using liquid semen, a minimum amount of 2.5 million total sperm per dose is required. Consequently, each ejaculate could provide 1 440 to 4 800 doses. However, when using frozen semen, approximately 20 million total sperm per dose are required. This would amount to 180 to 600 doses of frozen semen per ejaculate.” [18]. These are enormous numbers, and they help us better grasp the substantial numerics that are at stakes in the production animal industry.

In zoos, such technics allow researchers to conduct gene banking programs to better map the pool of genetic material present within the population of animals living in zoos and so help better preserve the diversity of this genetic pool. Conservation efforts also benefit from cryopreservation of gametes as they can then be used later on in artificial insemination to try preserve endangered species. Recently this took a striking turn with the first successful insemination and subsequent birth of baby lion cub in the Ukutula Conservation Center (UCC) in South Africa North’s West province. This was done with fresh semen and not with cryopreserved gemmes but still shows us the amazing prospecting future of the practice, as once the technic will be mastered, will allow us to greatly increase the conservation effort of this vulnerable specie [19].

Cryopreservation of semen in reptiles, however, is a whole different story, indeed, the lack of research makes any tryout at the endeavor incredibly hard and give rise to a number of problems very tricky to tackle, that in other more “classic” species would not be a problem.

Most of the knowledge related to the subject come from a few selected papers on selective species or from closely related ones like birds or amphibians [13]. Nonetheless, cryopreservation attempts have been recorded in a few species of reptiles, which I will expend on in the next few chapters.

3.1 Reasons for cryopreservation in reptiles and its status

Cryopreservation technology has become predominant in the reproduction of economically relevant production animal species, but in reptiles, it is widely unknown, even though threat of extinction may be as high as 50% in crocodile species and chelonians [20].

Human activity is largely the cause of this threat and reptiles, some being predatory animals, sometimes even occupying the top of the food chain suffer a great deal from it. Whether it is linked to habitat loss, decrease in prey and food availability or outright poaching [13]. These points make the argument for better research on the cryopreservation of reptilian gamete even stronger and call on us to do more. Indeed, while spermatozoa have been cryopreserved in a few species [15, 21], no offspring from cryopreserved spermatozoa have been reported, and the generation of live young from AI has only occurred in a small number of species produced with fresh semen [13].

Despite the current decline in numbers, reptile do not relish on an increase of research in Assisted Reproduction Technologies (ART), some attempts of artificial insemination and cryopreservation have been recorded but are unfortunately not enough to constitute a real research effort at standardizing any ART process for the group [13].

This lack of research can be explained by different factors. Indeed, the wide variety of reproductive anatomy and physiology makes any attempt at standardization of ART in reptile as a one group entity difficult if not impossible, forcing scientists to focus their research small aspects, with some deciding to focus on specific species like snakes [21] or lizards [15].

Cryopreservation attempts in themselves, have been tried out in the past, and gives us some insights on what to expect from cryopreservation of sperm cells, indeed, it would appear that the motility of the sperm is recovered with rates that are much lower than plasma membrane integrity after cryopreservation of the gametes [22].

Although conservation effort and other research project may be considered as the predominant factor in the need of increased trial for cryopreservation of semen and AI of reptiles in general, we shouldn’t devaluate the importance of the economic and farming

demand that exotic species might bring forward. Indeed, a world where 90% of all farmed animals are born following AI, species like crocodilian are next on the ladder for industrialization of their productions, in Australia alone during the 2006/2007 period, leather of high-grade quality and the meat (so called “exotic meat”) from saltwater crocodiles generated about $9 million of revenues. With never-ending demand for more luxurious goods more and more people wanting to try out exotic sources of meat, these numbers have most probably increased since then [22].

In conclusion, the use of frozen–thawed semen in reptiles presents several potential improvements. These include the reduction in risk of transmitting infectious diseases that occur when animals are in close contact, utilizing semen from genetically superior males and enabling breeding across different geographic locations. These benefits can be amplified when integrated with other reproductive biotechnologies, such as sperm sexing [22].

3.2 Cryopreservation steps

As we mentioned previously, sperm cells may be obtained by either collecting sperm right after it is “expelled from the body”, by cloacal massage, electro-ejaculation, and so on; or by taking out the internal reproductive organs and so, the sites of spermatozoa storage [15].

When taking out the reproductive organs, it is important to note that once placed in the preservative fluid, you should make multiple incisions in the tract. This is done so in order to allow the sperm cells to “swim out” of the tract and go into the semen extender media. While the epididymis should be opened as it is where mature sperm cells are stored, the dissection and subsequent opening of the vasa deferentia and ducts should also be performed as recent studies indicate that in reptiles, a large number of mature sperm cells are also stored in it making it a large storage place in itself [15].

Once the semen has been obtained or the internal reproductive organs taken out and placed in the media, it is crucial to place them in a semen extender, indeed the role of such solution is to protect the sperm from the environment and avoid damage, but also, most importantly to us, protect the cells during the freezing process. Indeed, cryopreservation of gametes requires intense freezing, most usually using liquid nitrogen which is measured below -195.8°C. Without it, the sperm cells are at risk of dying of and if the medium used is not the correct one, defect to the sperm post thawing may occur [38].

In order to protect the sperm cells, your crucial extender media can take on different chemical composition, for example we use solutions like TEST–yolk, egg yolk extender containing

TES and Tris [23], or other chemicals like “Triladyl®” which it self contains Tris, citric acid, sugar, buffers, glycerol, water, and antibiotics (tylosin, gentamicin, spectinomycin, lincomycin) [26].

In farm animals like cattle, buffalo, and pigs, the up-to-date protocols recommend the use of extenders that are based on 20% egg yolk. These egg-based extenders are routinely around the world, but we can also attribute them certain limitations based on the granules present in said egg yolk. While low density lipoproteins help protect the semen from damage by covering the sperm membrane during freezing and thawing, the high-density lipoproteins and minerals inhibit respiration of sperm cells and reduce their motility which will then alter the viability and use of the semen post-thawing [24].

As I have explained, most semen protector nowadays are composed of egg yolk to help protect the sperm from external and internal damages. But egg yolk-free semen extender also do exist for farm animals [28]. In rams, they have been compared and while the yolk based extender achieved a significantly greater post thawed motility, the egg-free one proved to be capable of protected sperm cells through the freezing process and that it could be used in the field [28].

In pet animals like the dogs, cryoprotectants are also used, indeed in dogs, extender containing TCG-EY (tris, citric acid, glucose + 20% egg yolk) extender are commonly used. It is worth noting that in the case of dogs, two technics are routinely used, with good outcome of sperm viability in their cases. Those technics are the so called conventional freezing (CF) and the ultra-rapid freezing (URF). [27]

Cryoprotectants may also be supplemented with extenders to further protect the sperm from damage during the whole process. Non-penetrating and penetrating cryoprotectants play a crucial role in safeguarding sperm cells from the physical and chemical damage induced by ice crystal formation. Non-penetrating cryoprotectants, like polymers, aid in vitrification, while penetrating cryoprotectants, such as sugars, help mitigate toxicity [24].

4. Existing attempts in reptiles

4.1 In lizard

While rare and under-researched, cryopreservation of reptilian sperm was attempted in recent years. And a particularly good example of that would be the trials in 2019 in the yellow

spotted monitor lizard (*Varanus panoptes*), indeed after obtaining sperm samples by mean of postmortem dissection, the sperm cells were lavaged out of the reproductive tract using Dulbecco'sphosphate-bufferedsaline (PBS;Ca^{++},Mg^{++} free) (Sigma-Aldrich, St. Louis, MO, USA). They were diluted 1:1 in either DMSO or glycerol in PBS to achieve a final concentration of 5% v/v, 10% v/v or 20% v/v [15].

The cooling down to liquid nitrogen was done in a "fast" fashion as they reached their final temperature at a −32.1˚C/minute rate, this was achieved by suspending the straws 5 cm above liquid nitrogen in a foam ice box [15, 22]. The straws contain the frozen sperm were taken out the following day for analysis, with a thawing protocol that consisted into immersing the samples into a 500 ml water bath at 35˚C for 1 minute [15]. This study resulted in an average post-thaw motility of 18.1%. This is much lower compared to sperm kept in 10% DMSO at room temperature, which had 57.5% motility, and unfrozen sperm, which showed 88.2% motility [15].

4.2 In snakes

Cryopreservation of sperm in snakes has also been attempted in the past. While not many attempts have been recorded, studies have tried to not only freeze retrieve live sperm cells and to determine the best combination cryoprotectant required for such exploits. A particular study in the Louisiana pine snake (Pituophis ruthveni) has even attempted to test the cryoprotective effectiveness of seventeen different CPA treatment mixtures aimed at preserving sperm viability and motility after thawing. Each mixture used a base diluent, either Lake's solution or TEST yolk buffer. The formulations included up to four membrane-permeating cryoprotectants: N,N-dimethylacetamide, N,N-dimethyl formamide, methanol, and glycerol. In addition, two non-membrane-permeating cryoprotectants, trehalose and fructose, were tested. Several additives were also incorporated, such as glycine, bovine serum albumin, fetal bovine serum, sodium lactate, and sodium pyruvate. Egg yolk, a widely used cryoprotectant for sperm, was included in all the treatment mixtures [21].

The highest total average motility was found to be 19.9% which very encouraging for future research and may pave the way for more tailored made protocols in snakes and other reptiles in general [21].

4.3 In crocodiles

Although AI has been successfully applied to the American alligator (*Alligator mississippiensis*), leading to the production of 11 fertile eggs [30], it has not yet reached the

level of effectiveness needed for widespread use in crocodilian farming to boost productivity. Additionally, it has not been successfully utilized to address infertility or promote genetic diversity in endangered crocodilian species [22].

This study conducted in 2013 by Johnston et al, provided us with the first comprehensive analysis of the physiochemical tolerance of crocodilian sperm, using spermatozoa from the saltwater crocodile (*Crocodylus porosus*), revealing several key findings: first and foremost, crocodile sperm can survive short-term manipulation in PBS diluent without egg yolk; secondly, they are able to tolerate rapid temperature changes without cold shock, even without egg yolk protection and the egg yolk does not improve short-term or chilled preservation; the spermatozoa were able to endure extreme hypotonic conditions. [22].

4.4 In Turtles and tortoises

While cryopreservation of sperm obtain from a member of the Testudines order was never attempted yet to the best of our knowledge, studies on how to better preserve its sperm has been done and documented. Different extenders were tested (Eel extender, Tyrode medium, phosphate buffer solution…) with various degrees of success [14].

The refrigeration medium test yolk buffer and the Tyrode medium supplemented with albumin, lactate and pyruvate) gave the best results and were able to successfully keep some cells alive for a period of 24h post sample collection, respectively 28-24% (depending on species) and 14% survival rate [14].

Materials and methods:

1. Collection of animals

Our first specimen was a 6 years old male veiled chameleon (*Chamaeleo calyptratus*), that had a planed euthanasia because its health was under the life standards, hence the requested euthanasia by the owner. Because of its age, we didn't know if our first sample could yield a large enough number of spermatozoa to be relevant in this study, we then decided to collect more specimen and of different species to try and compare and have a better understanding of the specific interspecies differences within the genus and not only evaluate the differences in cryoprotectant effects.

Our other speciemens were donated to the clinic a batch of chameleons coming from Madagascar. After inspection of the specimens, six males carpet chameleons (*Furcifer lateralis*) and three panther chameleons (*Furcifer pardalis*). All were adults and weighed 41g, 71g and 137g for the panther chameleons and 6g and 7g for the carpet chameleons. They had no concurrent disease that we know of. They were donated to the Department of Exotic Animal and Wildlife Medicine for dissection and to determine the cause of death which happened between 1 and 3 days prior, they had previously been stored in refrigerated conditions by the owner.

We decided to use these species as we thought they would serve a great basis for further research and studies. Indeed, they are very well represented species, both in the wild population and in captivity.

All animals were officially legged to the University of veterinary medicine of Budapest and Department of Exotic Animal and Wildlife Medicine and their respective owners agreed for us to perform euthanasia and/or dissection and subsequently to conduct research with the animals in accordance with the ethics of research, animal welfare and scientific standards in order to further scientific knowledge and development.

2. Sample collection

The samples were all collected following the same protocols, this was done to ensure that any change in the semen quality before and after the cryopreservation process was solely due to the differences in protocols or due to species differences and not due to different collection

methods. It is important to note however that different collection methods (that were mentioned and explained earlier) should be attempted in future research in order better understand the impact it would have on the sperm viability if any. These variations in viability may be caused by drug interaction, stress and other unknown parameters.

Upon reception and general examination of the animal in the clinic, the chameleons were put under anesthesia using 1mg/kg bodyweight meloxicam (Melovem 5 mg/ml oldatos injekció), 10mg/kg bodyweight tramadol (Tramvetol 50 mg/ml oldatos injekció kutyák részére A.U.V.) injected intramuscularly to the front leg and given 30 minutes waiting period. After that we induced anesthesia using alfaxalone (Alfaxan Multidose 10 mg/ml oldatos injekció kutyák, macskák és kedvtelésből tartott nyulak részére A.U.V.), at a dose of 5 mg/kg, injected intravenously in the tail vein. Approximately five minutes after the anesthetic injection, we made sure it didn't feel tactile or pain stimulus before euthanasia was performed by means of 0.1 ml intra-cardiac injection of T61 solution (T61 oldatos injekció A.U.V.), once again following standard euthanasia procedures.

When the heart of the chameleons had stopped and the animal was pronounced dead, we performed an opening incision to expose the internal organs. In order to get a better view and diagnose and obvious concurrent disease, we performed a large window of the coelom. The rib cage of the chameleons was removed, and we exposed the thoracic organs from the vertebrae to the sternum. The organs were exposed in a similar fashion, giving us an unobstructed view of the entire insides of the body cavities. The dissection was performed as followed: a first incision was made along the third intercostal space and a second along the spine from the third rib to the base of the tuber coxae. Two more incisions were made parallel to the first two to create a rectangle. Said rectangle was then dissected out to expose the internal organs.

Once we had a good view of the different organs and were able to identify and potential illnesses if any, we located the testicles and the rest of the reproductive apparatus, all found inside the body cavity in chameleons and other reptiles. The epididymis and the vas deferent were dissected out and placed in a PBS solution straight away to minimize any post-mortem damage. All chameleons that took part in this study had healthy genital tracts except the veiled chameleon who was found to have one of his testicles not being suitable due to an unidentified malformation. We decided to keep on with extraction of the second testicle and further sample analysis as this allowed us to practice and fine tune our technic as well as

getting a better idea of the morphology and anatomy of the spermatozoa of the veiled chameleon.

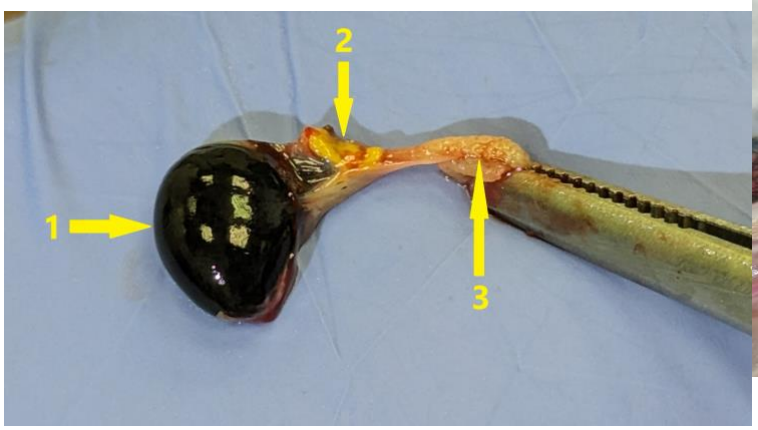


Figure 1: Removed genital tract of a male panther chameleon 1 testis, 2 epididymis, 3 vas deferens

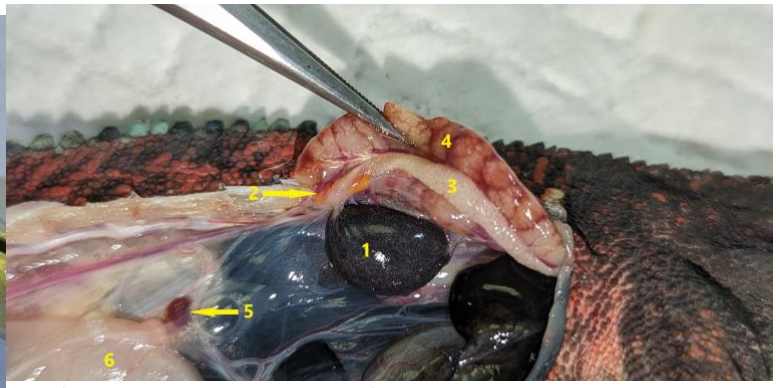


Figure 2: Panther chameleon with open coelom before take out the reproductive organs
1 testis, 2 epididymis, 3 vas deferens, 4 kidney, 5 spleen, 6 stomach

Live/dead rate:

The proportion of live cells in each sample was analyzed with the VitalScreen dye method, according to the manufacturer's protocol. At the first step, 50 µl of sperm sample was mixed thoroughly with 2 drops of Stain A and incubated for 30 seconds. Then, this solution was mixed with 3 drops of Stain B and incubated for an additional 30 seconds. Afterwards, a small drop of sperm-stain mixture was placed on a microscope slide and a smear was made using a cover glass. Colorless spermatozoa referred to as live, while red stained spermatozoa referred to as dead. At least 100 sperms was counted and viability rate was calculated.

Motility:

Since there is no standard method for chameleon motility analysis (neither manual nor computer assisted), short videos were captured. In the first step, 10 ul of sperm was put on heated microscope slide, covered with coverslip. 1-minute-long videos were made on 400X magnification (Olympus BX40 microscope with CMOS camera). Sperms were counted in each field of view, then the rate of non-motile, non-progressive and progressive motile sperms were calculated by two operators independently. The indicated rates are the means of two calculations. The same procedure was done on fresh and post-thaw samples, respectively.

4. Cryoprotectant used:

In this study, we decided to take commonly used cryoprotectants in the veterinary world as it could serve a base for further studies. Because our aim was to see how good the different solutions work would compared to each other, we needed multiple options, but we also wanted to limit ourselves in the number of solutions as using too many cryoprotectants would ultimately lead to too much confusion and make our data less interesting as well as less scientifically relevant.

For those above-mentioned reasons, we decided to use Biladyl®, Triladyl® and CaniPlus one-step®. These mediums were all manufactured by the company Minitube® and are used for varied purposes in our university lab when conducting research.

Two of the ones that we chose, namely the Biladyl® and Triladyl®, are usually and routinely used in the farm animal industry in bovine, ovine and other ruminant species while the third one, CaniPlus one-step®, is used in canine sperm cryopreservation.

Biladyl® is composed of egg yolk, buffers (such as Tris), glycerol, antibiotics, and sugars like glucose to preserve sperm viability; different antibiotics are added, including: tylosin, gentamycin, spectinomycin and lincomycin are part of the formulation. CaniPlus One Step® is a single-step extender containing glycerol, buffers, sugars, and other stabilizers but is free from egg yolk. Triladyl® includes egg yolk, TRIS, glycerin, antibiotics, citric acid, sugars and extremely pure water, making it suitable for multi-species sperm preservation.

5. Cryopreservation process

Once the spermatic cord and the epididymis were dissected out from the animals and placed in separate vials the samples obtained were immersed in 500 µl of PBS solution with 10% bovine serum for preservation. They were brought to the lab immediately after collection and they were placed on a heated plate set on 37.5 ˚C to keep the sample on this temperature.

Under the microscope, multiple incisions to the testis, epididymis and vas deferens were then performed and 15 minutes was given for the spermatozoa to settle and swim out of the reproductive tract into the solution.

After 5 minutes had passed for the sperm cells to swim out, we proceeded to the samples check of each individual to assess whether or not the sample quality was good enough to attempt cryopreservation. During this quality sample check, we controlled the gross number of sperm cells present in the sample and in case of sufficient number of spermatozoa, we assessed if there was enough motility. To do so, we pipetted out 10 µl from each sample and placed them of separate microscopic slides. Following this short analysis, were able to decide which samples were good enough to be frozen and which ones were not.

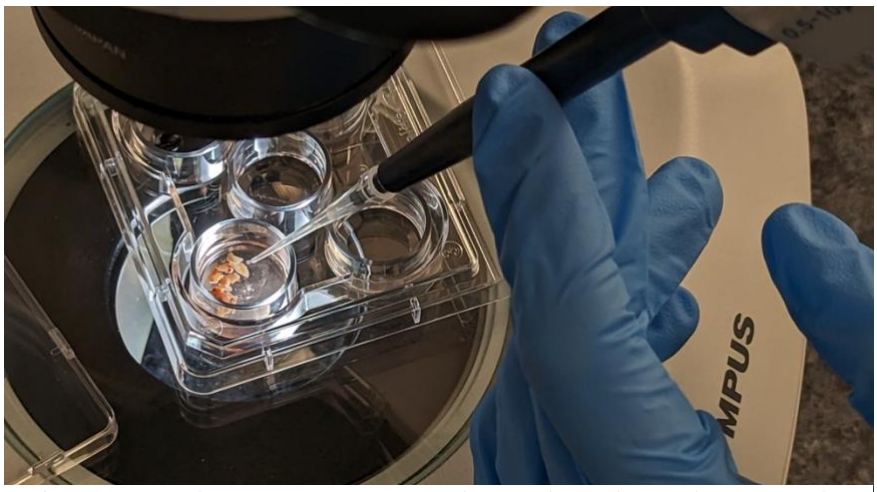


Figure 1: Collection of spermatozoa following 15 minutes incubation on warming plate

As mentioned before, the samples obtain from the veiled chameleon were deemed not to have the potential to be effectively cryopreserved as only few sperm cells, who were likely already dead, were retrieved during the quality check analysis.

During the second batch of chameleons, we were able to obtain a total of nine reproductive tract samples. From three panther chameleons and six carpet chameleons.

For clarity, named the samples by code with the letter “L”, “P” and “V”, respectively for “Lateralis”, “Pardalis” and “Veiled”; followed by a number to differentiate the different samples and keep precise record of the results. Form this we had V1, P1, P2, P3, L1, L2, L3, L4, L5, L5 and L6.

We were able to obtain four spermatozoa samples where enough motility was found on which we could attempt cryopreservation. One of those sample was from the panther chameleons and the remaining three were from the carpet chameleons. Results of the analysis are

recorded in table 1 with explanation on why the sample was included or excluded from the pilot study.

Table 1: Fresh Semen visual analysis

Sample ID	Used / Discarded	Cause
V1	Discarded	Low sperm count, low motility
P1	Discarded	Low motility
P2	Used	High sperm count, high motility
P3	Discarded	Low motility
L1	Discarded	Low motility
L2	Discarded	Low motility
L3	Used	High sperm count, medium motility
L4	Used	High sperm count, high motility
L5	Discarded	Low motility
L6	Used	High sperm count, high motility (highest of all samples)

After identifying which samples we would be attempting to cryopreserved, we had to add the cryopreservants medium that would hopefully protect the spermatozoa during the freezing process in liquid nitrogen.

We pipetted from each sample three times 100 µl into different eppendorf tubes giving us a total of 12 samples to be frozen (ie: 4 samples x 3 medium = 12 eppendorf tubes). To those twelve samples we added 100 µl of the different mediums. Following this process, we now had our 4 samples each mixed with three different conservation solution, Each with a 1:1 dilution ration of sample and cryopreservants.

In order to have the best rate of freezing and limit the risk of destroying the samples, we couldn't freeze the eppendorf tubes directly. Instead, we used thin plastic straws (0.25 ml ministraws), two for each sample. One was to be thawed during the next step of the pilot study while the other one was to be kept in liquid nitrogen for either further analysis or for later research projects.

The straws were then placed in the fridge for equilibration for 50 minutes before being suspended above liquid nitrogen for further ten minutes following what they would be immersed in it completely.

Once in liquid nitrogen, which temperature is at a constant -196˚C, they would be able to stay for a prolonged period of time without being further damaged.

Figure 2: Suspension of the straws 5cm above liquid nitrogen

6. Thawing process

After 5 weeks in liquid nitrogen, we thawed the samples and analyzed them. In order to do so, we once again used the same method for each of the sample.

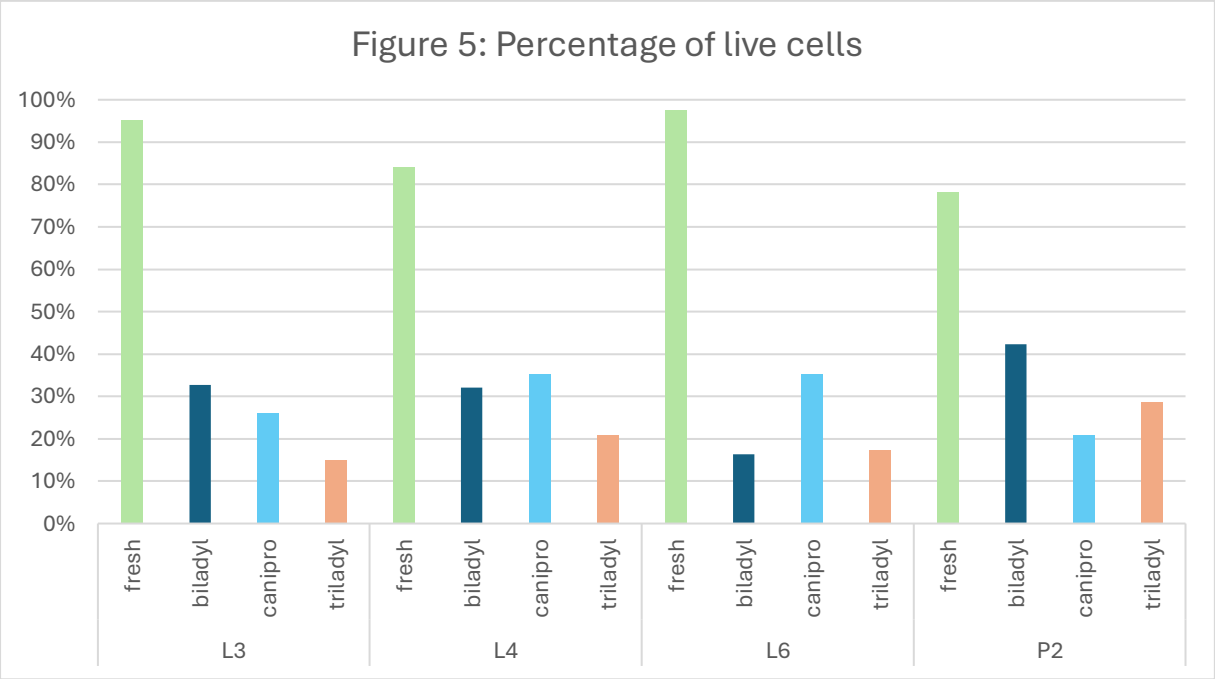
First, the sample was warmed from liquid nitrogen by placing it in a warm water bath at approximately 38-39˚C. Once thawed, the sample was transferred into a small Eppendorf tube. A 10-microliter portion was pipetted onto a glass microscope slide for analysis under the microscope. During this step, videos and/or photos were taken to document the findings. If sufficient viability was observed, the remaining portion of the sample was used to prepare two smears: one for assessing morphology and the other for evaluating vitality.

Following the thawing of the samples, we were able to analyze visually the different samples and assess whether or not some sperm cells had survived the freezing process and it’s subsequent thawing.

Results analysis

1. Live cell rate:

Once the samples were analyzed, we were able to draw out results for how the cryopreservants performed. The results are summarized in the following graphic (Figure 5).



We can see that while most of the spermatozoa did not survive the cryopreservation process, a non negligible amount did which is very encouraging results for us. We were not able to retrieve enough numbers to further the research in order to use the samples for ARTs, but this shows that it may be possible in the future as we continue to develop the technic.

1.1 Fresh Viability

From this analysis, we were able to confirm our choice in samples to be cryopreserved. Indeed the samples “L2, L5, P1, P3” had less live sperm count than the other samples. Our decision to exclude samples L1 and L5 during our initial analysis might have been unwise as they both exhibit good live rate upon examination. Motility however was not ideal hence our deviation to exclude the sample.From the samples that were used in the cryopreservation, all fresh sample selected had good live rate, with a minimum live percentage of 78% in the P2 sample and a maximum of 95% for L6. All results are summarized bellow in table 2.

Table 2: Fresh semen, live death percentages (nb: ID with an "" are the ones used in the cryopreservation)*

ID	Treatment	live	death
L1	fresh	91%	9%
L2	fresh	45%	55%
L3 *	fresh	95%	5%
L4 *	fresh	84%	16%
L5	fresh	83%	17%
L6 *	fresh	98%	3%
P1	fresh	46%	54%
P2 *	fresh	78%	22%
P3	fresh	95%	5%

1.2Post thaw viability

Once the samples were thawed, we proceeded with the same staining process for the analysis of the samples were cryopreservants was added. The findings of the visual examination are summarized in table 3 and the computerized analysis are shown in table 4.

Table 3: Post-thaw visual analysis of cryopreserved chameleon sperm

Sample	**Biladyl® media**	**Triladyl® media**	**CaniPlus® media**
P2	Post thaw motility present.	All dead	All dead
L3	Mostly dead cells but some alive ones	Mostly dead cells but some alive ones	Mostly dead cells
L4	Post thaw motility present	All dead	Mostly dead cells
L6	Post thaw motility present.	Mostly dead cells	Mostly dead cells

Table 4: Post-thaw viability rate of chameleon sperms in each treatment groups

ID	treatment	live	death
L4	Fresh	84%	16%
L4	Biladyl®	32%	68%
L4	Triladyl®	21%	79%
L4	CaniPlus®	35%	65%
L3	Fresh	95%	5%
L3	Triladyl®	15%	85%
L3	Biladyl®	33%	67%
L3	CaniPlus®	26%	74%
P2	Fresh	78%	22%
P2	CaniPlus®	21%	79%
P2	Triladyl®	29%	71%
P2	Biladyl®	42%	58%
L6	fresh	98%	3%
L6	CaniPlus®	35%	65%
L6	Biladyl®	16%	84%
L6	Triladyl®	17%	83%

From the following results, we can see that out of the three cryopreservants, Biladyl® and CaniPlus one-step® worked best while sample immersed in Triladyl® had the worst rate of survival. Biladyl® is a cryopreservants predominantly used in ruminant semen and it is very interesting to see that it was able to protect the semen up to 42% in the P2 sample, while its lowest performance was in the L6 sample with 16% of live cells recovered. CaniPlus one-step® a medium mostly used in canine cryopreservation, also had good outcomes with samples L6 and L4 both retaining 35% of live cells, L3 had 26% of live spermatozoa post thaw and P2 had 21% of live cells which was the lowest of the four samples.

Triladyl® was the cryoprotectant that had the lowest rate of survival, with a live percentage post thaw for P2, L3, L4 and L6 of 29%, 15%, 21% and 17% respectively. The medium, usually used in bull semen and other ruminants, was able to give us a few live cells post thaw, although less than the previous two mediums.

2. Motility

2.1Fresh motility

When analyzing the motility of the spermatozoa, we need to know what was before the cryogenic process, as it would give a baseline for analysis. We decided to only analyze the

samples selected previously for cryopreservation, whose results are summarized below in table 5.

Table 5: Fresh semen motility percentages

FRESH	%		
Sample	Progressive	Non-progressive	Non motile
L3	1	19	80
L4	15	15	70
L6	40	40	20
P2	10	60	30

2.2Post thaw motility

The results of the of the sample that were cryopreserved are summarized below in table 6.

Table 6: Post-thaw motility percentages

POST-THAW	%			
Sample	**Extender**	**Progressive**	**Non-progressive**	**Non motile**
L3	Biladyl®	0	1	99
	CaniPlus®	1	2	97
	Triladyl®	0	3	97
L4	Biladyl®	0	20	80
	CaniPlus®	0	1	99
	Triladyl®	0	0	100
L6	Biladyl®	5	15	80
	CaniPlus®	0	0	100
	Triladyl®	0	1	99
P2	Biladyl®	0	50	50
	CaniPlus®	0	0	100
	Triladyl®	0	0	100

The results we obtain were very interesting as they differ from the live/death percentile analysis with very few motile sperm (especially low in progressive motility), but also corroborate are findings in terms of which extender performed best. Indeed we can see that once again, Biladyl® and CaniPlus one-step® gave us the best results, with 50% of motility for P2 Biladyl®. CaniPlus one-step® and Biladyl® mediums were the ones that retained progressive motility, with L3 and L6 respectively (L3=1% and L6=5%).

Discussion/Conclusion:

Cryopreservation of chameleon sperm was both an incredibly interesting endeavor and tough research to conduct from start to finish. The lack of knowledge on the topic on some basic principles that wouldn't be a problem in mammalian species like spermatozoa morphology or known cryopreservants made the task a lot more challenging for the completion of this pilot study.

The results we obtained from the three cryoprotectant mediums were very encouraging and having a sample retaining 42% of live cells post thaw with the P2 sample with Biladyl® was a great surprise, especially considering the fact that it was corroborated by a post thaw motility of 50%. Overall, Biladyl® and CaniPlus one-step® gave us the best results whereas Triladyl® gave us the poorest performance.

However, it is important to note that although motility was found in 8 of the samples out of the original 12, only two of them had progressive motility, both at very love percentage (1% and 5%), which would render the different sample unusable for ART process. Also, the post thaw motility percentages were similar but lower than that of the live/dead analysis, meaning that while "alive", the spermatozoa were mostly non-motile. These findings are encouraging non the less and are liquefy due to the limitations we faced in this research, something we should aim at reducing for future endeavors.

Limitations we may have had included the facts that some of the samples had not enough sperm cells or motility, this may be caused by lack of production due to age or not the right reproductive period (we don't know enough on the reproductive cycle of the chameleons). Moreover, fine tuning the analysis methods, especially for the non-computerized ones like the live/death percentage count, is crucial in eliminating any human error. The lack of morphological knowledge also rises questions on the reproductive ability of the sperms.

Overall, when comparing the initial goals of the research and the results we got, we are very pleased and confident that this project can act as a solid foundation for the future. Through this study, we were able to prove that post-mortem sample collection can be successful in chameleons as well as doing so in properly stored carcasses that were 1-3 days old. Even more so, we were able to cryopreserve and successfully thaw the sperm cells from previously mentioned samples, which is an extraordinary achievement as we were the first to do it.

In the future, this research may be expended by obtaining a larger pool of samples, a larger number of species and testing new cryopreservative mediums. This we believe, may also serve as a basis for ex-situ breeding programs.

Abstract:

Cryopreservation offers significant potential for the conservation of genetic diversity, particularly in rare or endangered species such as chameleons. ARTs, or “Assisted Reproductive Technics” while common in mammals, are rare in reptiles and research on them is often not conducted as deemed less important, and/or less relevant. As far as we know, this is the first attempt to the cryopreservation of chameleon spermatozoa. Researches have been attempted on other reptilian species like the crocodilians and in snakes to try and lead the way to an increased level of research on this particularly interesting field of reptilian reproduction. But the lack of research on chameleons themselves made this study all the more challenging.

Little is known about the ideal protocols for successful sperm cryopreservation in these reptiles, even less in species like the ones treated in this study, namely the veiled chameleon (*Chamaeleo calyptratus*), the Carpet Chameleon (*Furcifer lateralis*) and lastly the panther chameleon (*Furcifer pardalis*). Little is also known on some basic reproductive informations of chameleons, such as the spermatozoa morphology.

This study aims to evaluate the efficacy of three commercially available extenders: Triladyl®, Biladyl®, and Caniplus® freeze one step, all manufactured by Minitube®; we aimed at evaluating the viability and motility of chameleon spermatozoa post-cryopreservation. Semen samples were collected postmortem from ductus spermaticus from cooled dead animals from captive and wild caught chameleons and subjected to cryopreservation using the respective extenders, followed by thawing and assessment of sperm viability and motility.

From the 9 animals we collected, 4 gave us good enough initial motility to try and proceed with cryopreservation of the samples. The mean live percentage of spermatozoa at collection (fresh) was 88.78 %, which is substantial enough to try the freezing process. It resulted in a mean live percentage post-thawing of 26.88% across all medium of preservation, with the Biladyl® and CaniPlus one step® performing best, each with a mean live percentage of 30.8% and 29.3% respectively. These results showed that not only the spermatozoa were able to survive the cryopreservation process but to an extent beyond expectation.

While this study has a number of positive outcomes which encourages us to do more research on the topic, limitation are to be noted. Indeed, no morphological analysis was performed, renders us unable to predict if the live spermatozoa that were recover would be fit for further reproductive uses. Nonetheless, these results should not be overlooked but rather used as a seeping stone for further research on the topic.

A krioprezerváció jelentős lehetőséget kínál a genetikai sokféleség megőrzésére, különösen a ritka és veszélyeztetett fajok, például a kaméleonok esetében. Az asszisztált reprodukciós technikák (ART) gyakoriak az emlősöknél, azonban ritkán kerülnek alkalmazásra hüllőkben. Az általunk megvizsgált szakirodalmi adatok alapján ez az első kaméleon spermiumok mélyhűtésére irányuló kutatás. Más hüllőfajokkal folytattak már ez irányú kutatásokat, például krokodilok és kígyók esetében. A kaméleonokkal végzett vizsgálatok eddig ismeretlen területe még nagyobb kihívás elé állította a projektet.Keveset tudunk a hüllő spermiumok sikeres krioprezervációjának ideális körülményeiről, még kevesebb információ áll rendelkezésre az általunk vizsgált fajok tekintetében. A kísérletben résztvevő fajok spermium morfológiájáról is minimális információ áll rendelkezésre. A kutatás során három faj spermiumát fagyasztottuk, majd olvasztottuk ki: sisakos kaméleon (*Chamaeleo calyptratus*),szőnyegkaméleon (*Furcifer lateralis*) és párduckaméleon (*Furcifer pardalis*). Kutatásunk célja három kereskedelmi forgalomban kapható spermahigító hatékonyságának összehasonlítása: Triladyl®, Biladyl® és CaniPlus Fr eeze®, amelyeket a Minitube® gyárt. Céljaink közt szerepelt a kaméleon spermiumok életképességének és mozgékonyságának értékelése a mélyhűtést megelőzően és azt követően. A spermiumoktenyésztett és vadon fogott kaméleonokból származtak. A mintákat post mortem, hűtött tetemek ductus spermaticusábólgyűjtöttük, és a megfelelő spermahigítókkal mélyhűtöttük, majd felolvasztást követően értékeltük a spermiumok életképességét és mozgékonyságát.A vizsgálatba 9 egyedet vontunk be, melyből 4 egyed mintái mutattak megfelelő motilitást ahhoz, hogy elvégezzük a minták előkészítését és mélyhűtését. A mintagyűjtést követőenaz élő spermiumok aránya átlagosan 88,78% volt, melyet megfelelőnek ítéltünk a fagyasztási eljárás alkalmazásához. A kiolvasztás után az élő spermiumok aránya átlagosan 26,88% volt mindhárom hígítót tekintve. Vizsgálataink alapján a Biladyl® és a CaniPlus Freeze® teljesített a legjobban, az ezzel kezelt minták sorrendben 30,8% és 29,3% élő spermiumot tartalmaztak átlagosan. Ezek az eredmények nemcsak azt mutatják, hogy a spermiumok képesek túlélni a krioprezerváció folyamatát, de azok felolvasztás után is alkalmasak lehetnek további felhasználásra.Noha ennek a kutatásnak számos pozitív eredménye van, amelyek további kutatásra sarkallnak bennünket a témában, meg kell említeni a limitációit is. A kaméleon spermiumok morfológiája pontosan nem ismert, így ezen vizsgálatok alapján nem mondható ki, hogy a kiolvasztott és életben lévő spermiumok alkalmasak lesznek-e további reprodukciósfelhasználásra. Mindazonáltal ezek a kaméleon spermiumokkal kapcsolatos első vizsgálati eredmények jó alapot biztosíthatnak a jövőbeni kutatások számára.

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