ABSTRACT

Title of Dissertation:	INVESTIGATING SPERM MATURATION DURING EPIDIDYMAL TRANSIT IN THE DOMESTIC CAT FOR THE DEVELOPMENT OF ASSISTED REPRODUCTIVE TECHNIQUES
	Tricia Marie Rowlison, Doctor of Philosophy, 2017
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Understanding the physiological processes involved in the sperm maturation process is critical to elucidating mechanisms underlying male infertility. Maturation of the spermatozoa during epididymal transit is associated with the integration of specific proteins and acquisition of functionality. Many underlying processes are unclear thereby making it challenging to develop applications for assisted reproductive technologies. The goal of this research was to understand key phases in the sperm maturation process associated with functional effects of protein incorporation via epididymosome secretion on sperm cell motility and fertilization. Specific objectives were to (1) identify essential proteins that contribute to sperm centrosome maturation throughout epididymal transit, (2) characterize the transfer of key factors via epididymosomes between the epididymis and maturing sperm cells and (3) demonstrate the impact of such mechanisms on the acquisition of motility and fertilizing ability by

the spermatozoa. Using the domestic cat model (*Felis catus*), we demonstrated that the secreted epididymal vesicles termed epididymosomes supply critical proteins to the developing spermatozoa and examined the effects of exposure of these vesicles on motility and ability to successfully fertilize an oocyte. Exposure of immature sperm cells to epididymosomes *in vitro* resulted in the significant incorporation of cenexin, critical for the maturation of the sperm centrosome, which then enables proper pronuclear syngamy and formation of the first mitotic spindle following oocyte penetration. Furthermore, this research focused on assessing the impact of malformed spermatozoa at the centrosome on fertilization success in the domestic cat. Findings revealed that improper maturation of the centrosome may be a source of infertility in this species as well as in wild felids. In summary, this research has provided insights into the sperm cell maturation process which will enhance the use of assisted reproductive techniques and reproductive success of many species including the human and wild felids.

INVESTIGATING SPERM MATURATION DURING EPIDIDYMAL TRANSIT IN THE DOMESTIC CAT FOR THE DEVELOPMENT OF ASSISTED REPRODUCTIVE TECHNIQUES

by

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

This dissertation is dedicated to my parents, Deborah and Kendall Rowlison.

Acknowledgements

I would like to thank my committee members, Drs. Mary Ann Ottinger, Pierre Comizzoli, Carol Keefer, Adrienne Crosier, and William Bowerman for their time and assistance. All of my committee members provided valuable support and feedback which helped me grow throughout my graduate school experience. I would like to thank Drs. Crosier and Bowerman for serving on my committee as they provided significant knowledge and expertise of wildlife conservation, which helped me broaden the scope of my dissertation and keep in mind the "big picture" of my research. I would also like to personally thank Dr. Keefer for lending her expertise of reproductive physiology which helped me overcome many challenges of my project, and for pushing me personally to grow as a scientist. With her quick wit, kindness, and remarkable knowledge, she inspires me as a scientist.

I especially would like to thank Dr. Ottinger for her continued support throughout my graduate school experience. She accepted me as a graduate student at a time when she herself was transitioning positions and was soon retiring from UMD. She nonetheless agreed to be the chair of my committee and has offered her full support throughout my entire program. She helped me achieve many accomplishments and has been invaluable to my success as student and scientist.

I would like to give my sincerest thanks to Dr. Comizzoli who made this experience possible. He accepted me into his lab at the Smithsonian Conservation Biology Institute and helped me coordinate my degree with Dr. Ottinger through the University of Maryland. Throughout my program he has served as a wonderful mentor

and aided me tremendously in the completion of my studies. The knowledge gained from his advisement has immensely assisted my development as a scientist in the field of reproductive physiology and wild animal conservation.

I would also like to thank my fellow graduate students who supported me through my program. My labmates at SCBI- Dr. Pei-Chih Lee, Dr. Lara Mouttham, Dr. Gina DellaTogna, Dr. Tamikia Phillips, Morgan Dundon, Kate Wilsterman, my labmates at UMD- Dr. Kara Duffy and Dr. Meredith Bohannon, and to everyone else that I had the wonderful opportunity to meet and work with. I would also like to give a big thanks to the interns that assisted me in completing my project: Bridget Aylward, Kylie White, Connie Brinks, Sarah Sampliner, and Sarah Jacobs. Everyone made my experience enriched and joyful and I hope for the opportunity to someday collaborate with you again. I would also like to thank my fellow graduate students that I met through the Animal Sciences Graduate Student Association (ASGSA). The club supported me both financially and emotionally as we all progressed through our graduate careers. I thank you all deeply and will cherish the fun times that we got to share with one another.

To my best friends, Marie Iwaniuk and Leslie Juengst, I can never thank you enough for all your love and support that you have given me through these years. Whether it was through sharing some beers at Hard Times, the encouraging messages to get me through a rough day, or all the fantastic experiences that we shared, I could always count on you. Even when I lost faith in myself, you were my biggest supporters and helped me to believe in myself once again. It wasn't always easy being miles away

from my home, but I truly felt like I had a family here with you. You are both such wonderful, funny, and brilliant ladies. Stay beautiful.

I would also like to thank my friends and family back home for supporting me throughout my entire life and many (many!) years as a student. I especially would like to thank my brother, Mitch Rowlison, for all his support and encouragement. It is truly wonderful to have a best friend that I can also call my brother. I would also like to thank my grandmother, Dee Brown, for all the times that she provided me love and support through our long-distance phone calls.

Last, but foremost, I would like to give my biggest thanks to my parents, Deb and Ken Rowlison. I dedicate this dissertation to you as I would not be here without you. You have given so much love and support that I can never thank you enough. You encouraged me to pursue my dreams no matter what and have been so supportive every step of the way. From when I was that crazy kid picking up insects to when I decided to pursue my Ph.D. you have both had such faith in me that I knew I could achieve anything. I truly thank you, and love you both very much.

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Chapter 1: Introduction and Literature Review

Assisted reproductive techniques for the conservation of endangered species

The use of assisted reproductive techniques has become crucial for managing fertility of wild animals and improving the conservation of endangered species. These are especially important for managing captive breeding populations which serve as an *ex situ* reservoir to hedge against extinction in the wild (Pukazhenthi et al., 2005). These populations have become indispensable as the conservation of land is becoming ever more difficult to maintain due to the increasing human population and expanding urban sprawl. Maintaining a healthy *ex situ* population allows the possibility of animal reintroductions, research, and serves as a source of inspiration for the general public which may lead to increased interest in supporting conservation efforts.

Assisted reproductive techniques may include a number of procedures such as gamete collection- whether it be through semen collection or gamete retrieval such as in the case of a recently diseased individual, *in vitro* gamete maturation and fertilization, hormonal monitoring and synchronization, artificial insemination, and cryopreservation. Each of these can serve as a valuable tool for the reproductive success of a species (Pukazhenthi et al., 2005; Wildt et al., 2010; Comizzoli et al., 2010). However, many challenges are faced

when attempting to use these techniques due to individual or species-specific complications. Enhancing our knowledge of the reproductive physiology of both wild and domestic species is imperative for the successful application of assisted reproductive techniques to animal conservation.

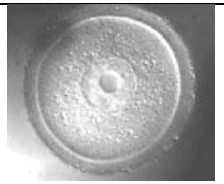
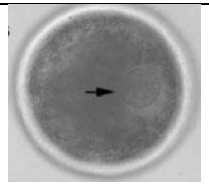
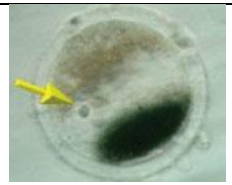
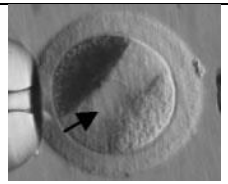
Use of the Domestic Cat as a Model Species

The research conducted for this study used the domestic cat as a model as it is a close relative for wild felids. Of the 38 wild felid species, 18 are classified as 'vulnerable' or 'endangered' (IUCN Red List, 2017). Previous research has demonstrated that the more commonly used animal models may not be the best fit for wild felids as some reproductive mechanisms significantly differ. For example, a study that explored the use of artificial insemination in the cheetah (*Acinonyx jubatus*) did not have success when applying a technique that was established in the bovine model (Wildt et al., 2001). While species-specific variations may limit the use of the domestic cat as a model for wild felids, it remains the most suitable option for the study of feline physiology and the development of assisted reproductive techniques.

Additionally, the domestic cat shares many similarities in its reproductive physiology with humans which makes it valuable biomedical model. A comparative study between model species demonstrated that the domestic cat is significantly more similar to the human in oocyte morphology and development (Table 1.1; Comizzoli et al., 2008; Wildt et al., 2010). Because of these similarities, the domestic cat has provided vital information

regarding female infertility including asynchronous oocyte maturation, and hypersensitivity and dysfunction of the ovary following gonadotropin therapy (Comizzoli et al., 2010). Furthermore, the male domestic cat serves a valuable model for the study of teratospermia- a condition in which the majority of sperm cells (≥ 60%) exhibit morphological abnormalities (Pukazhenthi et al., 2001; Neubauer et al., 2004), which has been observed in a number of species including human and wild felids (Pukazhenthi et al., 2006).

Table 1.1. Comparative morphology and *in vitro* maturational biology of immature oocytes in five mammalian species (Wildt et al., 2010).

	Mouse	Cow	Pig	Cat	Human
Morphology of an immature oocyte[a]					
Oocyte diameter (µm, excluding zona pellucida)	80	110	125	110	110
Germinal vesicle diameter (µm)	30	35	35	45	45
Nuclear configuration	Large nucleolus; clumped chromatin	Small nucleolus; clumped chromatin	Small nucleolus; filamentous chromatin	Small nucleolus; fibrillar chromatin	Small nucleolus; fibrillar chromatin
Meiosis achieved in vitro (hr)	<24	~24	~44	~24	~24
Reference	Liu and Aoki (2002)	Lodde et al. (2007)	Sun et al. (2004)	Comizzoli et al. (2008)	Combelles et al. (2002)

[a]Pig and cat oocytes were centrifuged to polarize cytoplasmic droplets to visualize the germinal vesicle (arrow).

The domestic cat has served as a useful model for exploring a number of techniques in fertility preservation. This includes studies of cryopreservation such as the effects of cryoprotectants on sperm cells (Pukazhenthi et al., 2002) and oocytes (Comizzoli et al., 2004), cooling rates of sperm cell cryopreservation (Pukazhenthi et al., 1999, Pope et al., 1991; Zambelli et al., 2002), and cryopreservation of oocytes (Murakami et al., 2004; Luvoni and Pellizzari, 2000; Luvoni et al., 1997), preantral follicles (Jewgenow et al., 1998), and gonadal tissue (Mouttham et al., 2016; Hovatta et al., 1996). Additional studies have also reported an alternative approach to the preservation of oocyte germinal vesicles via a microwave-assisted drying technique which bypasses the need for cryopreservation and allows for storage at room temperature, providing an easier and more feasible option for gamete preservation (Elliot et al., 2015). Studies of other assisted reproductive techniques using the domestic cat have included artificial insemination (Platz et al., 1978; Axnér et al., 2002; Tsutsui et al., 2000 and 2003), *in vitro* fertilization (Johnston et al., 1989; Lengwinat et al., 1994; Spindler and Wildt, 1999; Karja, 2002), embryo transfer (Pope et al., 1993; Swanson, and Godke, 1994; Gómez et al., 2003), xenografting of testicular tissue (Snedaker et al., 2004; Kim et al., 2007), and somatic cell nuclear transfer (Kitiyanant et al., 2003; Yin et al., 2006).

While these studies have advanced the use of assisted reproductive techniques for felid species, there are still many challenges that limit success. Improving the understanding of basic physiology in the domestic cat as a model

will not only enhance the use of these techniques, but may also contribute to new translational approaches for wild felid fertility preservation and conservation. In particular, studies on spermatozoa structure and function are needed due to challenges in cryopreservation and assisted reproduction resulting from the high incidence of teratospermia in felids. Investigating the maturation process of the spermatozoa in the domestic cat will assist in overcoming these challenges and improve breeding management of wild cat species.

Spermatogenesis and the Sperm Cell Structure

The main structures of the sperm cell consist of the head, midpiece, and flagellum (Figure 1.1, as reviewed by Senger, 2005). The sperm head contains the nucleus comprised of the male's DNA, and the acrosome cap which functions during the acrosomal reaction to bind with the oocyte zona pellucida and allow for sperm penetration. The midpiece connects the head to the flagellum of the sperm cell. This section contains the centrosome which functions after fertilization to produce the sperm aster - a mass array of microtubules which bind to both male and female pronuclei and facilitates pronuclear fusion (Schatten et al., 1994; Palermo et al., 1997). The midpiece also contains a number of mitochondria which serve to produce ATP (adenosine triphosphate), the energy substrate required for sperm motility. Last, the

flagellum, or tail, is comprised of axial filaments which propel the sperm cell through the female's reproductive tract and aids in penetrating the oocyte.

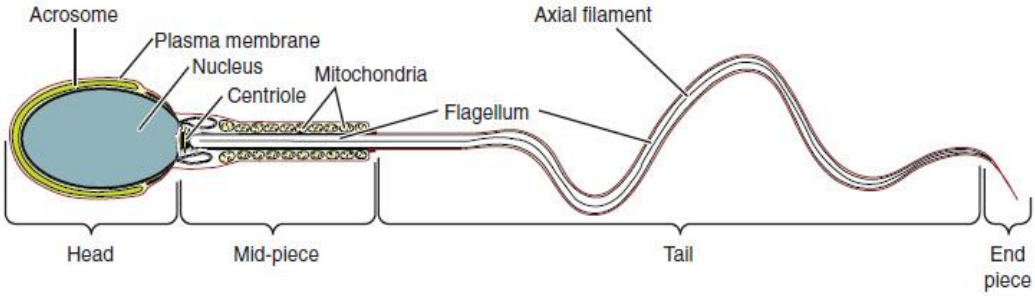


Figure 1.1. Diagram of the sperm cell.

Spermatozoa are first produced in the testis segment of the male's reproductive tract (Figure 1.2; Encyclopedia Britannica, 2008) and are termed, "spermatogonia" (Senger, 2005). These are formed by spermatogonial stem cells, which line the basement membrane of seminiferous tubules and undergo a series of mitotic and meiotic divisions, aided by the proteins and hormones supplied by the Sertoli cell. This process is termed, "spermatogenesis", and forms the round spermatid at completion (Figure 1.3; Cooper 2012). While the spermatid has completed the majority of its morphological development, further maturation within the epididymis is required to achieve functionality and the ability to successfully fertilize an oocyte (Mescher, 2011).

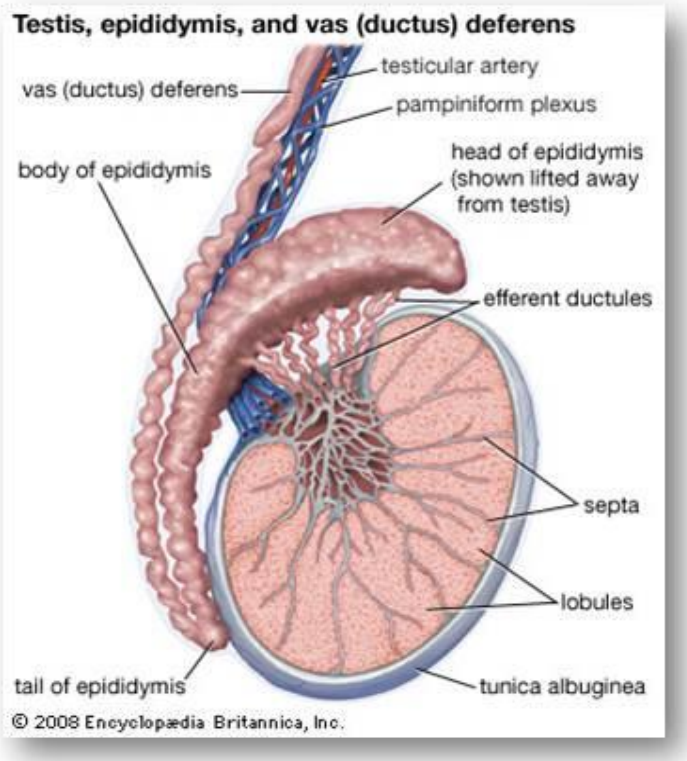


Figure 1.2. Diagram of the mammalian testis (Encyclopedia Britannica, 2008).

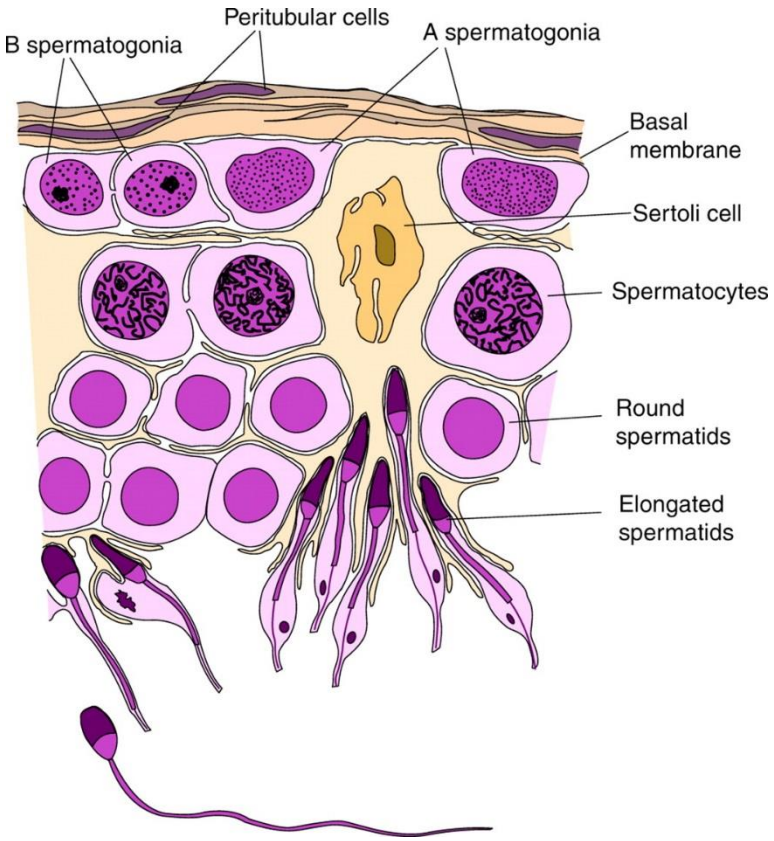


Figure 1.3. Diagram depicting spermatogenesis (Emaze, 2017).

Sperm Cell Maturation

In mammalian species, the spermatid will undergo further maturation following spermatogenesis via transit through the epididymis. This results in a sperm cell population that is able to achieve motility, capacitation, sperm-egg binding, and fertilization (Senger, 2005; Cooper 2012; Dacheux and Dacheux, 2014). The epididymis is a relatively long tubule which consists of three main segments: the caput (head), corpus (body), and cauda (tail; Figure 1.4; Fàbrega et al., 2011). Transit is completed in one to two weeks in the mammalian species studied to date. During this time, the sperm cell will undergo a series of physiological changes which are dependent upon the proteins, enzymes, and other various factors that are supplied by the surrounding epididymal epithelial tissue.

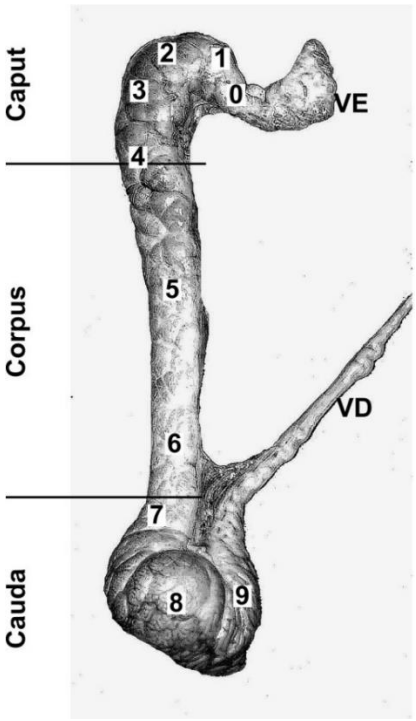


Figure 1.4. Diagram of boar epididymis. Caput= 0-4, Corpus= 5-6, Cauda= 7-9. VE= vas efferens (efferent ducts), VD= vas deferens (Fàbrega et al., 2011).

During the early stages of epididymal transit, a large amount of the luminal fluid (80-90%) is reabsorbed. This not only increases the sperm cell concentration, but also results in an increase in the protein concentration (Dacheux et al., 2012). For example, analysis of the stallion and bull luminal fluid demonstrated a change in cell concentration from 10^8 spermatozoa/mL in the rete testis to 10^9 in the vas deferens, and the protein concentration from 2 to 4 mg/mL in the rete testis to 50–60 mg/mL in the caput segment (Fouchecourt et al., 2003; Belleanne et al., 2011). The sperm cell will also undergo initial changes which include migration of the cytoplasmic droplet, a remnant of the cytoplasm that formed during the earlier stages of spermatogenesis, and will begin to acquire the abilities to move and bind with the zona pellucida. However, these functional capabilities will not be fully achieved until the spermatozoa completes the entire epididymal transit (Dacheux and Dacheux, 2014).

The sperm cell will acquire its ability to become motile through a series of modifications that occur throughout the epididymal transit. *In vivo*, the sperm cell will remain mostly quiescent throughout transit of the whole epididymis; however, *in vitro* studies have observed weak motility beginning with sperm cells isolated from the caput segment which were washed free of the epididymal fluid and incubated in commercial media that induces motility (Bork et al., 1988; Chevrier and Dacheus, 1992). At this stage of maturation, the flagellum exhibits limited and irregular beating and is unable to move in a forward direction. As the sperm cell continues its transit, it will acquire maturational

proteins and exhibit an increase in intracellular cAMP (cyclic adenosine monophosphate, a derivative of ATP; Dacheux and Dacheux, 2014; Turner, 2005). The increase in cAMP will form a signaling pathway involving protein kinase A (PKA) which will subsequently allow the sperm cell to achieve greater motility and hyperactivation- the ability to produce a high amplitude, asymmetric tail beat which allows the spermatozoa to pass through the cervical canal and uterine cavity and penetrate the zona pellucida.

Once the sperm cell reaches the oocyte, it must be able to bind and penetrate the zona pellucida and fuse with the oocyte plasma membrane (Figure 1.5). The ability to achieve this is also acquired throughout epididymal transit. As the sperm cell approaches the oocyte, it will undergo the acrosome reaction in which the acrosomal cap fuses with the sperm plasma membrane, allowing for the release of the acrosome contents (Senger, 2005). Hyaluronidase is a key enzyme, which is supplied by the epididymis. This enzyme will enable the spermatozoa to progress through the cumulus cell complex which surrounds the oocyte and is comprised of hyaluronic acid (Martin-DeLeon, 2006). Hyaluronidase breaks down the gel-like structure of the expanded cumulus cell complex via hydrolysis, thereby allowing the sperm cell to reach the zona pellucida.

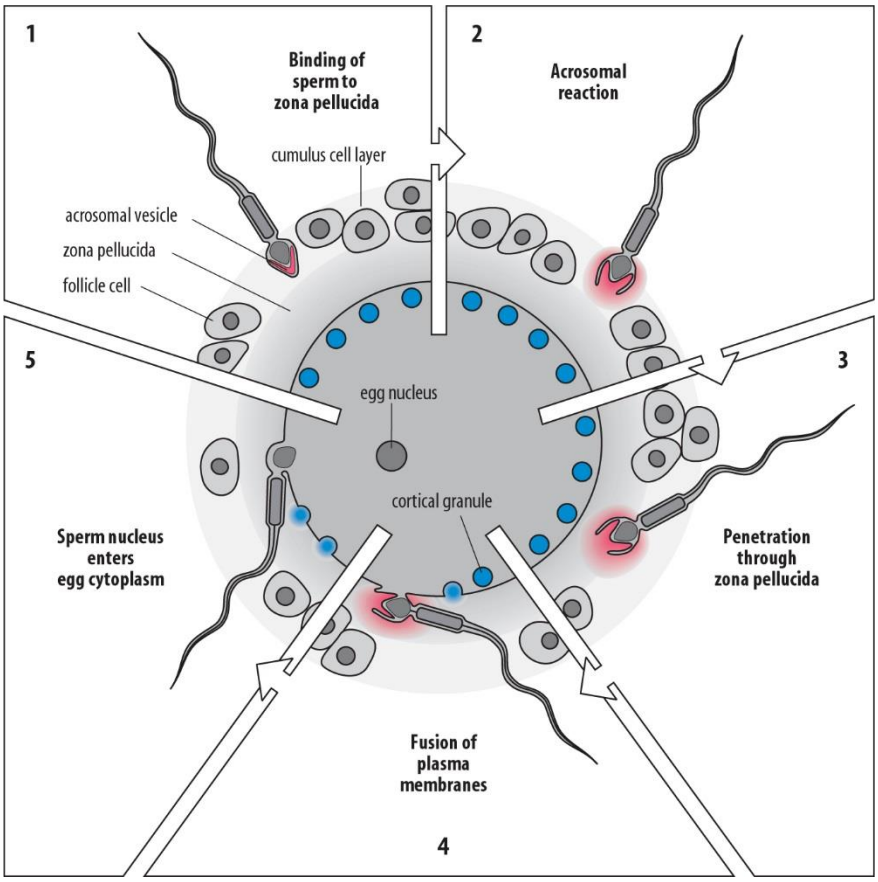


Figure 1.5. Steps to fertilization: 1= binding of sperm cell to zona pellucida, 2= acrosome reaction, 3= zona pellucida penetration, 4= plasma membrane fusion, 5= incorporation of sperm (Munca, 2017).

Subsequent binding with the zona pellucida is reliant on various proteins, two of the most notable being IZUMO, and ADAM3 (A disintegrin and metalloprotease 3; Dacheux and Dacheux, 2014). While the acquisition of IZUMO has not been identified within the epididymal tract, ADAM3 has been observed to be secreted by the epithelial tissue and incorporated by the maturing sperm cell. Both proteins have been identified as markers for fertility as spermatozoa which lack these factors exhibit a significant decrease in the ability to bind and penetrate the zona pellucida. Upon penetration, the sperm cell is then able to fuse with the oocyte plasma membrane and complete the subsequent steps of early development.

The Sperm Centrosome

The sperm centrosome is an essential organelle playing a key role just after penetration into the oocyte. It serves to organize the sperm aster which is required for proper syngamy and formation of the first mitotic spindle (Schatten et al., 1994; Palermo et al., 1997). Located at the base of the sperm head, the centrosome consists of two centrioles termed the "proximal" and "distal" centrioles. In somatic cells, their counterparts are referred to as the mother (mature) and daughter (immature) centrioles, respectively. Centrioles are typically cylindrical-shaped and consist of 9 sets of microtubules symmetrically arranged in triplets, averaging 0.5 μm in length and 0.2 μm in diameter (Robbins et al., 1968; Vorobjev et al., 1982; Manandhar et al., 2005). There is a cloud of

pericentriolar material (PCM) which constitutes the microtubule organizing center (MTOC) of the cell. The PCM is comprised of over 100 different types of proteins that support microtubule growth which aids in numerous cell functions in the somatic cell including cell polarity, genomic stability, and cellular division (Lingle et al., 1998; Pihan et al., 1998; Hinchcliffe et al., 2001; Khodjakov et al., 200; Manandhar et al., 2005).

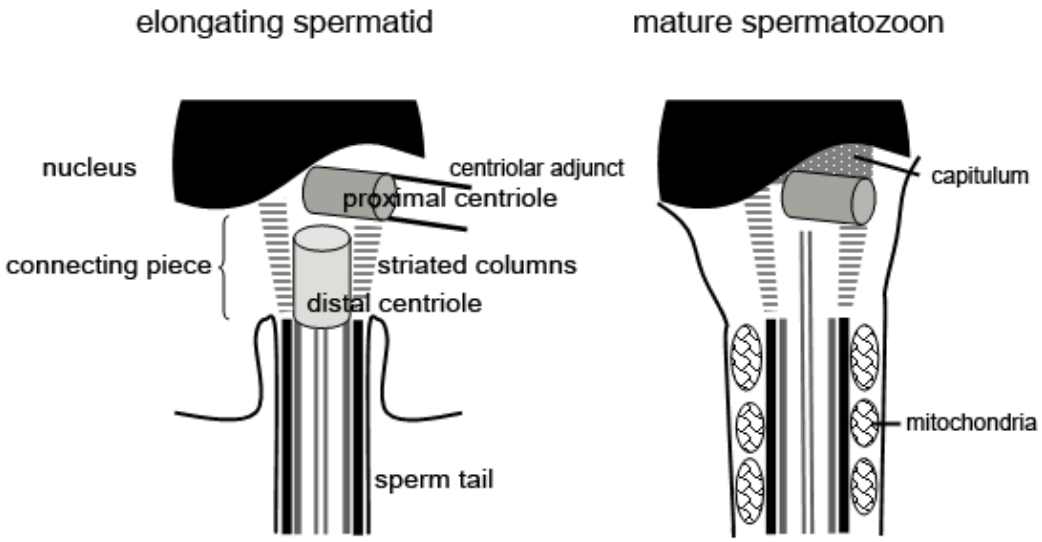


Figure 1.6. Diagram of the mammalian sperm centrosome (Atlas of Genetics and Cytogenetics in Oncology and Haematology, 2017).

As the sperm axoneme develops, the PCM becomes replaced by striated columns comprised of outer dense fiber proteins and the capitulum - a dense, disk-shaped structure, which together serve as a protective shield for both of the centrioles (Figure 1.6; Hoyer-Fender, 2011). The proximal centriole is positioned at a right angle and is surrounded by the striated columns whereas the distal centriole will assume a new role in forming the axoneme. In some species, such as the mouse (*Mus musculus*), the proximal centriole will disintegrate prior to fertilization; however, retention of the proximal centriole throughout sperm maturation has been confirmed using transmission electron microscopy in a number of species including the domestic cat (*Felis catus*) and tiger (*Panthera tigris*; Schmel and Graham, 1989), human and rabbit (*Homo sapiens,* and *Oryctolagus cuniculus*; Zamboni and Stafanini, 1971), little brown bat (*Myotis lucifugus*; Fawcett and Ito, 1965), Russian hamster (*Phodopus sungorus*; Fawcett, 1975), bovine (*Bos Taurus*; Sathananthan, 1997), and sheep (*Ovis aries*; Crozet et al., 2000) suggesting its function in forming the sperm aster after fertilization.

Following oocyte penetration, the proximal centriole is released from the sperm cell along with the male nucleus, and together, these migrate to the center of the embryo. Upon its release into the oocyte cytoplasm, the centriole will recruit necessary proteins to achieve full functionality (Stearns & Kirschner, 1994; Felix et al.,1994; Simerly et al., 1994). These proteins become available as a result of the degeneration of the oocyte centrosome during the meiotic stages of development (Figure 1.7; Manandhar et al., 2005). The

proteins are dispersed throughout the cytoplasm and are subsequently recruited by the sperm centrosome following penetration to aid in the production of a mass array of microtubules which are collectively referred to as the sperm aster (Schatten, 1992; Schatten & Schatten, 1986; Manandhar et al., 2005). The female pronucleus is then physically carried along the aster microtubules to the centriole via dynein motor proteins (Payne et al., 2003). As the female pronucleus is drawn close, the pronuclear envelopes which encapsulated the male and female chromatin disintegrates allowing for fusion of the genetic material and subsequent embryonic development (Figure 1.8).

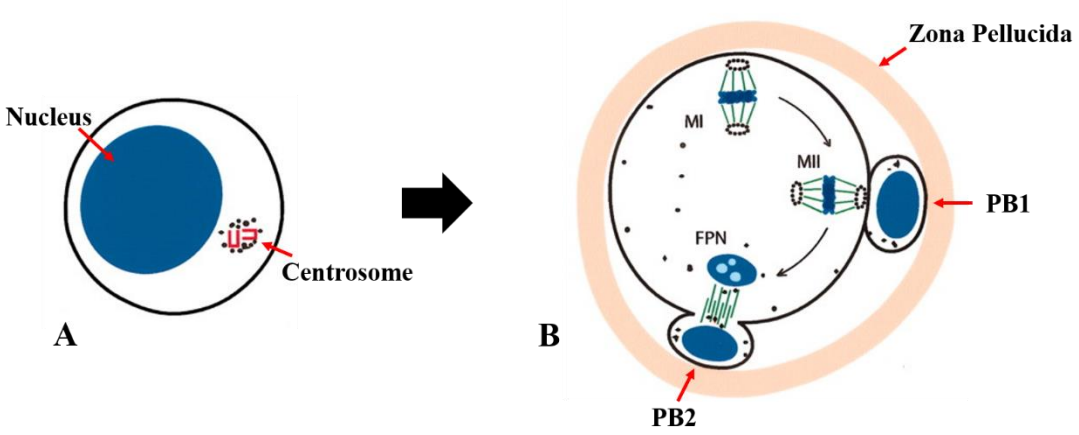


Figure 1.7. Centrosome reduction during oogenesis. A= oogonia containing centrosome with centrosomal proteins, B= centrosome reduction during stages of meiosis. M1 and M2= metaphase 1 and 2 nucleus, FPN= female pronucleus formation following meiotic divisions, PB1 and PB2= polar bodies 1 and 2. Blue= nucleus, green= microtubules, black dots= centrosomal proteins (adapted from Manandhar et al., 2005).

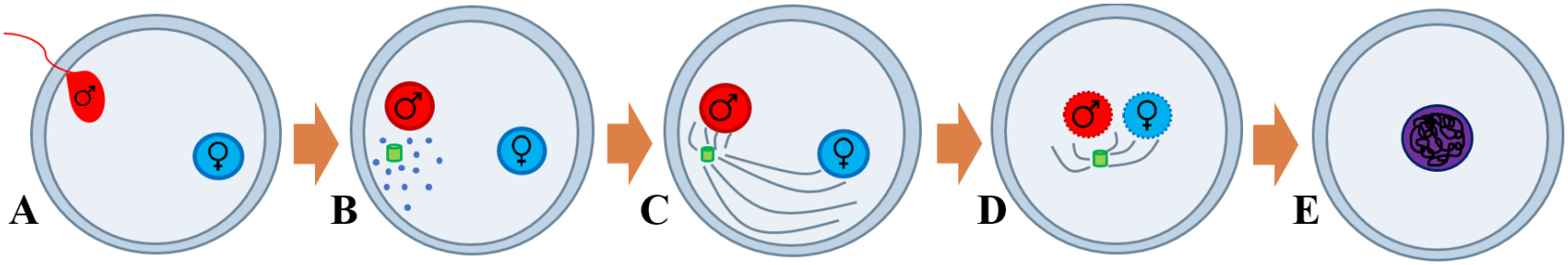


Figure 1.8. Sperm aster formation and pronuclear fusion. A= sperm penetration, B= release of centrosome and male nucleus into oocyte cytoplasm and recruitment of centrosomal proteins (blue dots), C= aster formation by centrosome, D= breakdown of pronuclear envelopes, and fusion of male and female pronuclei, E= fully fused zygotic nucleus.

Implications for Fertilization

Comizzoli et al. (2006) conducted a series of experiments which demonstrated the importance of centrosome maturation by injecting domestic cat oocytes with either mature, ejaculated spermatozoa or immature spermatozoa collected from the testis segment. Oocytes injected with mature spermatozoa resulted in the formation of a large, competent sperm aster. Conversely, injection of immature spermatozoa resulted in small asters and significantly increased time for the onset of first cleavage and embryonic death (Figure 1.9). These resulting differences indicated that there are key maturational processes taking place specifically for the centrosome while the whole sperm cell is undergoing maturation within the male reproductive tract.

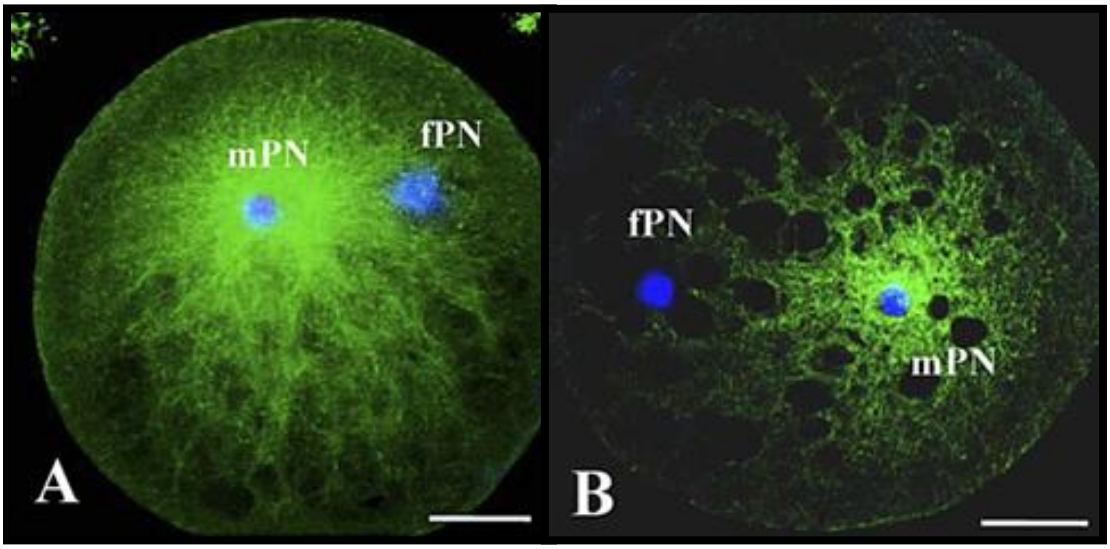


Figure 1.9. Images of aster formation (green) following fertilization with mature (A) versus immature spermatozoa (B); mPN, fPN= male, and female pronuclei respectively. Scale bar= 30 µm (Comizzoli et al., 2006).

This study also provided evidence that a dysfunctional centrosome may contribute to infertility. To show that it is necessary to have a fully mature and functional centrosome Comizzoli et al. (2006) conducted a second experiment in which oocytes were injected with the head of mature spermatozoa and a centrosome replaced with that of an immature spermatozoa. This also resulted in the formation of small asters, increased time for the onset of first cleavage and likelihood of embryonic death, therefore indicating that if an oocyte is fertilized with a mature, ejaculated sperm it is still possible to have impaired embryonic development if the centrosome is incompetent.

Comizzoli et al. (2006) also reported that it was possible to rescue the function of an impaired centrosome. Testicular sperm centrosomes, which were previously shown to result in the formation of small asters, were replaced with that of ejaculated sperm. This resulted in large, more competent aster formations which functioned similar to what was observed when oocytes were injected with whole, ejaculated sperm cells. Based on these results it was hypothesized that this technique could be used to improve the success of assisted reproduction in individuals which display this type of infertility. If a particular species, or individual, is shown to have a significant portion of its sperm display impaired centrosome function then it may be possible to replace the organelle with that of a fertile domestic cat donor's thus allowing for the valuable individual's genetics to be passed on to the next generation. While

promising, studies of this technique in wild cats have yet to be explored to the best of the author's knowledge.

Infertility is a major health concern that affects numerous species. Studies of the sperm centrosome and subsequent aster have increasingly identified related pathologies as possible causes of infertility in humans. Previous research has demonstrated correlations between sperm centrosome-derived pathologies and infertility in men. Based upon research first conducted in the bovine (Navara et al., 1996), Rawe et al., (2002) performed an assay to assess the centrosome function of infertile men via its formation of the aster after injecting human sperm into bovine oocytes. Staining for microtubules demonstrated poor aster formation which likely led to the observed developmental arrest that subsequently occurred. In contrast, sperm injected from a fertile donor resulted in the formation of large sperm asters and further embryonic development.

Other research in human fertility has shown that the functionality of the centrosome may be gauged through the assessment of sperm morphology. In a study conducted by Chemes et al. (1999), it was observed that a significant proportion of sperm cells collected from infertile men displayed abnormalities in the head-tail junction where the centrosome is located (Figure 1.10). The flagellum in these sperm types appeared to be misaligned and displayed increased fragility wherein the flagellum would readily dissociate from the rest of the cell. Selecting for sperm with properly aligned head-tail junctions of an infertile male's collection increased the likelihood of proper aster formation,

pronuclear fusion, and cleavage, indicating improved function of the centrosome. It is possible that the same type of morphological abnormalities may exist in other species and additionally be correlated with centrosome functionality. Previous reports have observed that morphological abnormalities of the whole midpiece region that encompass the head-tail junction have been commonly observed in many felid species (Pukazhenthi et al., 2006). Thus, it is possible that abnormalities specific to the head-tail junction may be present the spermatozoa of wild cats as well which could subsequently hinder fertilization success.

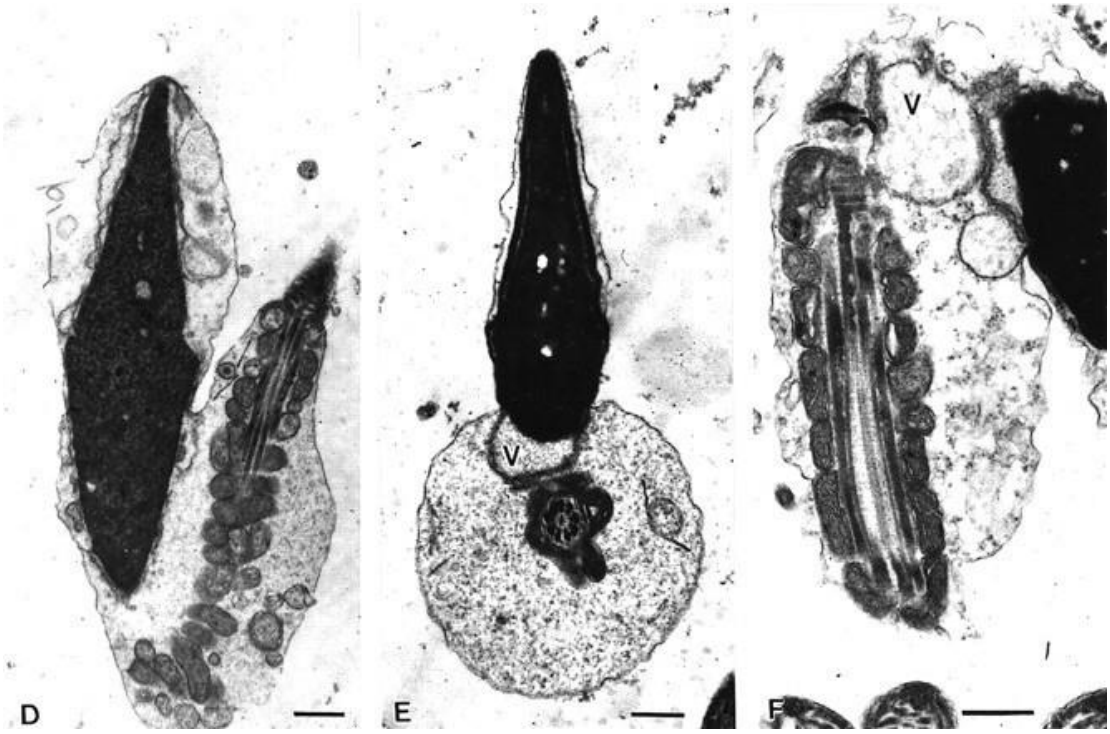


Figure 1.10. Infertile human sperm with abnormal head-tail alignment (edited from Chemes et al. 1999). (D) Abnormal alignment and connection with midpiece. (E & F) Abnormal alignment at head-tail junction with vesicular bodies (V; Chemes et al., 1999).

Centrosome-Associated Proteins

While the importance of a fully functional sperm centrosome has become increasingly evident, the maturational process for this organelle still remains relatively elusive. Although there are numerous proteins that bind with the centrosome throughout its role as the MTOC, there are only a select number that have been identified to associate specifically with the organelle to aid in its maturation and function (Schatten and Sun, 2009; Table 1.2). A number of these proteins are hypothesized to be secreted by the male's reproductive tract and incorporated by the centrosome while the whole sperm cell is undergoing its maturational process.

Table 1.2. Previous literature investigating centrosome-associated proteins

Protein	Cell Type/ Species	Function	Author
Cenexin	F9 cells (mouse)	Scaffold for other proteins (ninein and centriolin) at subdistal appendages. These appendages allow for microtubule anchoring and binding with cell cortex.	Ishikawa et al., 2005
Ninein	HeLa (human) and HCT116 (mouse) cells	Organizes microtubules by regulating nucleation and anchoring processes. Serves as a molecular link for γ-tubulin ring complexes.	Delgyhr et al., 2005
γ-tubulin	Xenopus	Binds with ninein to nucleate and anchor microtubules through the formation of ring complexes.	Zheng et al., 1995
Centriolin	C. elegans	Prevents premature centrosome dissociation from cell cortex and microtubule formation.	Schmidt et al., 2009
NuMA	HeLa (human) and CV-1 (monkey) cells	Anchors microtubules into aster formations.	Gaglio et al., 1995
Hook2	RAW 264.7 and CAD (mouse) cells	Binds with centriolin to aid in the nucleation and anchoring of microtubules.	Szebenyi et al., 2006
Speriolin	Human, mouse	Binds to human sperm centrosome (periphery of disintegrated distal centriole in mouse) and hypothesized to subsequently aid in the nucleation and anchoring of sperm aster microtubules.	Goto et al., 2010
End-Binding protein 1	COS-7 (monkey)	Aids in the nucleation and anchoring of microtubules.	Askham et al., 2002

One key protein that has been identified to aid in the maturation process is termed “cenexin”. Cenexin is a protein product from the same gene that transcribes sequences for the outer dense fiber 2 (ODF2) protein such that Odf2 and Cenexin are splice variants of each other (Huber, 2007). Originally thought to be the same protein, there have been differences observed in their expression, localization and function. It was not until 2007 that Huber et al. elucidated the genetic variation. While cenexin has been shown to be expressed in most somatic cells, ODF2 is primarily limited to the testis tissue of the male. ODF2 has been demonstrated to surround the sperm flagellum and functions to maintain its flexibility and protect against the sheer forces created during movement (Burfeind and Hoyer-Fender, 1991). Conversely, cenexin has been shown to localize to the subdistal appendages of the mature centriole within somatic cells (Lang and Gull, 1995). Only the mature, mother centriole plays a functional role in the MTOC. It is not until cellular division that the centrosome undergoes semi-conservative replication and the daughter centriole matures to a functional state. Because cenexin localizes strictly to the mature centriole, it has served as a maturational marker for the centrosome.

To assess the function of cenexin/ODF2, Salmon and coworkers (2006) analyzed the effect of inhibiting expression by knocking out the cenexin/ODF2 gene through a gene trap vector. Subsequent mating of heterozygous mice resulted in no homozygous offspring (as assessed at the e3.5, e6.5, e8.5, and blastocyst stage). This indicated that the homozygous embryos were unable to survive due to the loss of cenexin/ODF2 function. It is important to note that

the murine sperm centrosome naturally degrades as the sperm cell completes its maturation and is therefore not passed on to the zygote. However, maternally-derived centrosomes do form during early embryogenesis and these proteins are shown to play a role in the subsequent development. It is therefore possible that these proteins perform key tasks during the centrosome's early function which could otherwise render it incompetent.

Using cenexin-deficient F9 cells, Ishikawa et al. (2005) further discerned the function of this protein as it relates to the MTOC. Other proteins investigated included ninein, and centriolin (also referred to as CP110) which have both been shown to localize to the distal regions of the matured mother centriole suggesting these proteins may also play a role in centrosome maturation (Ou et al., 2002). Inhibiting expression of cenexin not only resulted in the lack of the protein's localization to the distal regions of the mother centriole (presumably the subdistal appendages), but also the localization of ninein and centriolin. This suggests that cenexin serves as a scaffold for these proteins. Surprisingly though, the formation of microtubules and cellular replication did not seem to be effected as the authors expected. Instead it was observed that the centrosome was unable to bind to the cell membrane and produce primary cilia as the organelle normally would, thus indicating that these proteins may together function in the docking process of the centrosome with the cell membrane which is necessary for its subsequent role in the formation of primary cilia. The proteins could also serve to prevent the premature production of the sperm aster until after the embryo extrudes the

second female pronucleus. Premature aster formation in *Caenorhabditis elegans* has been reported to cause the improper production of an additional meiotic spindle and result in chromosomal dysregulation and embryonic death (McNally et al., 2012).

There are a number of proteins that have been observed to play a role in the somatic cell centrosome regulation. The proteins listed in Table 1.2 refer to ones which may also potentially aid in sperm aster formation and regulation. While the functions of some have been studied in a variety of somatic cells, much remains unknown about their role in the sperm cell. Furthermore, it is also unknown how most of these proteins are incorporated into the centrosome while the sperm cell is undergoing maturation in the male reproductive tract. Investigating these processes will not only improve our understanding of the centrosome but may also enhance male fertility preservation options in humans and endangered species.

Protein Expression and Secretion

Identifying the expression and secretion mechanisms of key proteins secreted within the epididymis will also improve understanding of the sperm maturation process. As reviewed by Dacheux and colleagues (2003; 2014), the luminal fluid within the epididymis is dynamic as its composition changes between the consecutive segments. While analyses of the fluid have been conducted in a number of species including the stallion (*Equus ferus caballus*;

Fouchecourt et al., 1999), boar (*Sus scrofa*; Syntin et al., 1996), ram (Gatti et al., 2005), and human (Thimon et al., 2007), the identification of proteins is not complete. This is due to the limited detection of small proteins and the difficulty in attaining adequate separation of acidic, basic, and hydrophobic proteins.

The classical method of protein secretion is the merocrine pathway. Proteins expressed by this mechanism contain signaling sequences and are secreted individually (Nicander and Malmqvist, 1977; Dacheux and Dacheux, 2002). Once secreted into the luminal fluid the proteins then bind with the sperm surface and are integrated by the cell **(**Kirchhoff and Hale, 1996; Cooper, 1998; Sullivan, 1999). Another method of secretion has also been identified in a number of species including the rat (*Rattus rattus*; Fornes et al., 1995), hamster (Legare et al., 1999), bovine (Frenette and Sullivan, 2001), human (Thimon et al., 2007) and cat (Figure 1.11; Morales and Cavicchia, 1991). Proteins without a signaling sequence have been observed to be secreted within membranous vesicles termed, "epididymosomes". These vesicles are secreted from the apical pole of the epididymal epithelial cells in an apocrine manner (Hermo and Jacks, 2002). This mechanism does not include post-translational fusion of the individual protein with secretory signals; instead, the proteins form blebs at the apical pole of epithelial cells which detach and are released into the luminal fluid. Though the exact mechanisms of how these proteins are incorporated by the sperm cell are uncertain, it has been demonstrated that the epididymosomes will fuse with the outer plasma membrane to allow for

subsequent uptake of the proteins (Figure 1.12; Saez et al., 2003). In the bovine, the protein content of these vesicles was observed to differ from the proteins in the surrounding luminal fluid and also from epididymosomes secreted from other segments of the epididymis suggesting that the proteins secreted are incorporated by the sperm in a sequential manner (Figure 1.13; Girouard et al., 2011).

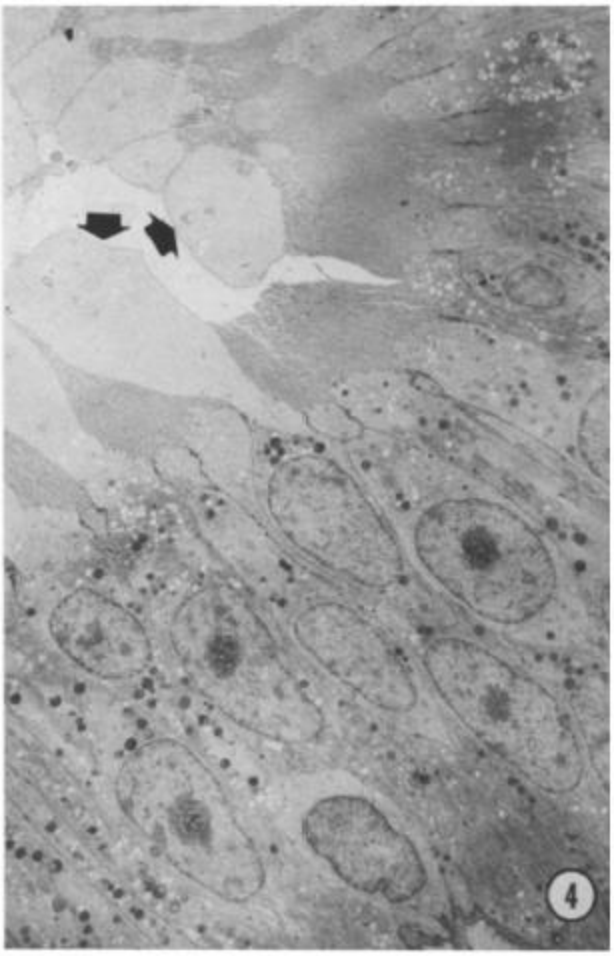


Figure 1.11. Domestic cat epididymal epithelium displaying secretion of epididymosomes (arrows) from the apical pole of principal cells, light microscopy x 2000 (Morales and Cavicchia, 1991).

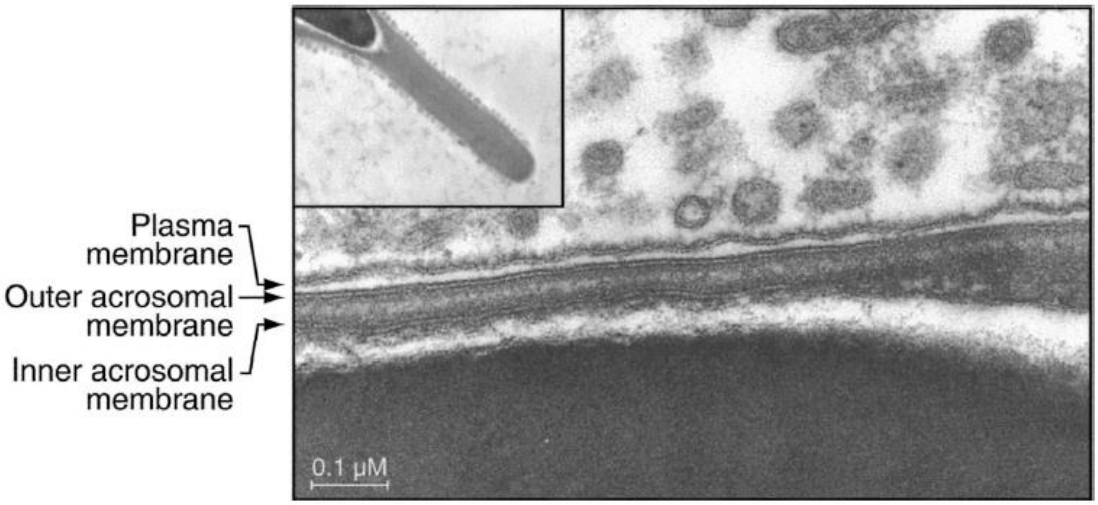


Figure 1.12. Electron photomicrographs of Chinese hamster epididymosomes surrounding the plasma membrane of a sperm cell. Inset= image of acrosome surrounded by the epididymosomes (Saez et al., 2003).

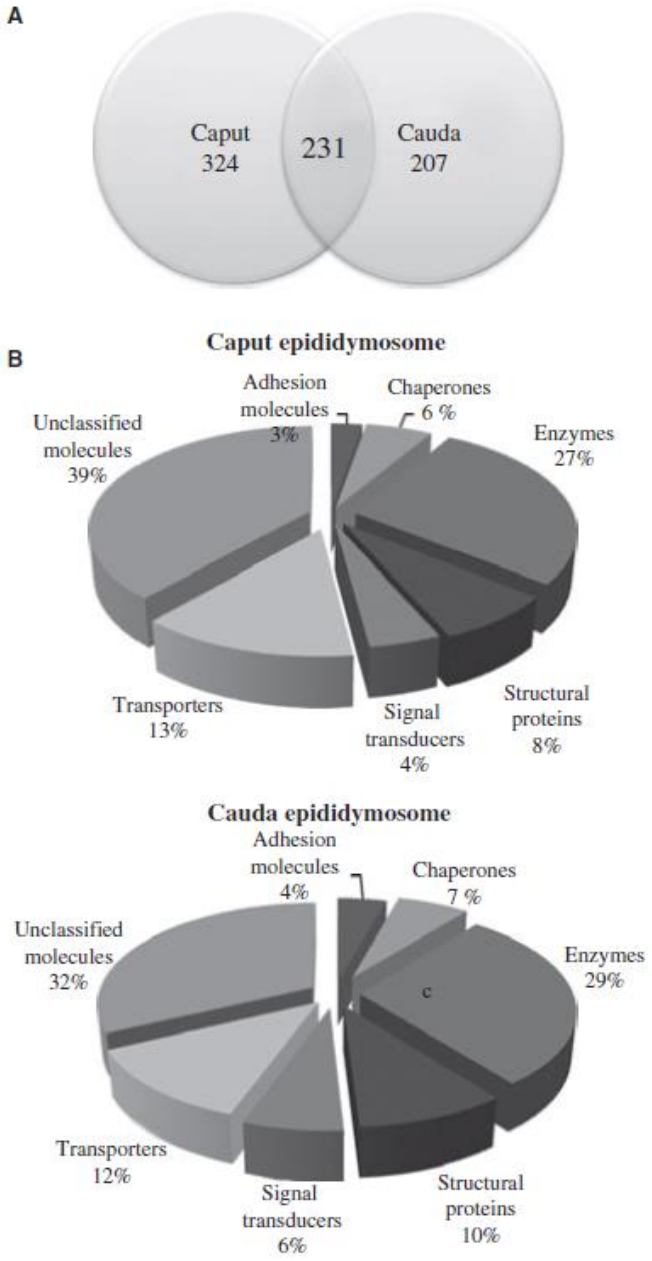


Figure 1.13. A) Identified epididymosome-associated proteins isolated from caput versus cauda epididymis in the bovine (analyzed via tandem mass spectrometry), B) Distribution of proteins according to predicted molecular functions (Girouard et al., 2011).

Similar structures termed, “prostasomes”, have also been observed in the seminal plasma and found to serve a role in the acquisition of functional traits such motility, and ability to undergo capacitation (as reviewed by Frenette and Sullivan, 2003). Conversely, potential effects of epididymosome exposure on these functions are not fully understood. In 2007, Sullivan et al. suggested that it is likely that the epididymal vesicles may promote sperm motility as they observed significant incorporation of multiple factors which modulate motility including aldose reductase, sorbitol dehydrogenase, and macrophage migration inhibitory factor (Table 1.3).

Epididymosomes have also been identified to transport proteins which aid in the sperm-egg binding (Table 1.3). For example, the proteins, P34H, P26h, and P25b have been identified to be associated with epididymosomes in the human, hamster, and bovine, respectively (Sullivan, 1999). These proteins are derived from orthologous genes between these species and function in zona pellucida recognition and binding during the acrosome reaction. Similarly, glutathione peroxidase 5 has also been identified as a protein which associates with epididymosomes in the mouse and serves to prevent premature acrosomal reaction (Rejraji et al., 2002). Tables 1.3 and 1.4 provide a summary of various proteins that have been previously identified to be associated with epididymosomes. Table 1.3 describes proteins which have been reported to aid in sperm cell motility and sperm-egg binding, and Table 1.4 describes proteins which function in other various cellular processes.

Table 1.3. Epididymosome-associated proteins that aid in sperm cell motility and sperm-egg binding.

Protein	Species	Function	Author
P34H, P26h, P25b	Human, hamster, bovine	Zona pellucida recognition and binding	Sullivan, 1999
A Disintegrin and Metalloproteinase (ADAM) 3A, and 1	Bovine	Zona pellucida recognition and binding	Girouard et al., 2011
Synthenin	Bovine	Zona pellucida recognition and binding	Girouard et al., 2011
Heat shock Protein 90 (HSP90)	Bovine	Zona pellucida recognition and binding	Girouard et al., 2011
Macrophage migration inhibitory factor	Rat, bovine	Sperm metabolism and motility	Frenette et al., 2003; Eickhoff et al., 2001; Girouard et al., 2011
Adenylate kinase isoenzyme	Bovine	Sperm metabolism and motility	Girouard et al., 2011
Aldose reductase	Bovine, ram	Sperm metabolism and motility	Frenette et al., 2003; Girouard et al., 2011; Gatti et al., 2005
Sorbitol dehydrogenase	Bovine	Sperm metabolism and motility	Frenette et al., 2003; Girouard et al., 2011

Table 1.4. Epididymosome-associated proteins that aid in various sperm cell functions.

Protein	Species	Function	Author
Ubiquitin	Bovine	Elimination of defective sperm cells	Sullivan, 1999
Epididymal Sperm Binding Protein (ESPB1)	Bovine	Elimination of defective sperm cells	D'Amours et al., 2012
Peroxiredoxin 1, 2, 4, 6	Bovine	Protection from oxidative damage	Girouard et al., 2011
Glutathione S-peroxidase P	Bovine	Protection from oxidative damage	Girouard et al., 2011
N-ethylmaleimide-sensitive factor Attachment Protein Alpha (NAPA)	Bovine	Intracellular trafficking	Girouard et al., 2011
Annexin	Ram	Intracellular trafficking	Gatti et al., 2005
GPX5	Mouse	Prevents premature acrosomal reaction, and protects membrane from lipid peroxidation damage	Rejraji et al., 2002
Phosphoethanolamine binding protein (PEPB)	Bovine, Ram	Membrane remodeling during acrosomal reaction	Girouard et al., 2011 Gatti et al., 2005
Thioredoxin	Bovine	Flagellum stabilization via disulfide bonding	Frenette et al., 2003; Girouard et al., 2011
Glucose-regulated protein (GRP-78)	Bovine	Sperm cell attachment to oviductal epithelium	Girouard et al., 2011

While progress has been made in understanding these maturational processes and acquisition of sperm cell functionality, much remains to be investigated. The number of identified proteins which are secreted within the epididymal tract has increased as proteomic technologies have advanced (Girouard et al., 2011; Gatti et al., 2005); however, many are still unidentified. Furthermore, it is also unknown how most of these proteins are incorporated by the sperm cell throughout maturation. Species-specific differences in the proteins secreted and mechanisms of incorporation also remains to be investigated in many species which are not the more commonly used animal models.

Study Aims

The overall aim of this research was to improve the understanding of the sperm centrosome maturation process and investigate the functional effects of protein incorporation via epididymosome secretion on sperm cell motility and fertilization. The significance of proper sperm morphology and motility has been demonstrated in both wild and domestic cats, particularly on the subsequent effects of abnormalities associated with decreased male fertility (Pukazhenthi et al., 2006). The effect of improper centrosomal development

has yet to be investigated as a possible contributing factor to the infertility in felid species. Furthermore, many of the key factors which drive changes in the sperm centrosome to acquire its developmental potential after fertilization have yet to be identified across many species. And while progress is being made to determine the composition of the epididymosomes secreted throughout the male reproductive tract, the influence of these vesicles on the acquisition of sperm motility and fertilization has not yet been demonstrated. Improving our understanding of the sperm centrosome maturation process and the effects of epididymosomes on the acquisition of sperm cell functionality will provide essential information for enhancing existing assisted reproductive technologies and developing new applications to increase the reproductive success of endangered species.

The objectives of this study were to (1) identify key epididymal proteins that contribute to the centrosome's maturation throughout sperm transit, (2) characterize the transfer of key factors via epididymosomes between the epididymis and maturing sperm cells and (3) demonstrate the impact of such transfer on the acquisition of fundamental properties by the spermatozoa.

References

Askham, J. M., Vaughan, K. T., Goodson, H. V., & Morrison, E. E. (2002). Evidence that an interaction between EB1 and p150Glued is required for the formation and maintenance of a radial microtubule array anchored at the centrosome. Molecular biology of the cell, 13(10), 3627-3645.

Bork, K., Chevrier, C., Paquignon, M., Jouannet, P., & Dacheux, J. L. (1988). Flagellar motility and movement of boar spermatozoa during epididymal transit. Reproduction, nutrition, developement, 28(5), 1307-1315.

Burfeind, P. and S. Hoyer-Fender (1991). Sequence and developmental expression of a mRNA encoding a putative protein of rat sperm outer dense fibers. Developmental biology, 148(1): 195-204.

Chemes, H. E., Puigdomenech, E. T., Carizza, C., Olmedo, S. B., Zanchetti, F., & Hermes, R. (1999). Acephalic spermatozoa and abnormal development of the head–neck attachment: a human syndrome of genetic origin. Human reproduction, 14(7), 1811-1818.

Chemes, H. E. Sperm centrioles and their dual role in flagellogenesis and cell cycle of the zygote. The centrosome, Springer: 33-48.

Chen, Z., Indjeian, V. B., McManus, M., Wang, L., & Dynlacht, B. D. (2002). CP110, a cell cycle-dependent CDK substrate, regulates centrosome duplication in human cells. Developmental cell, 3(3), 339-350.

Chevrier, C., & Dacheux, J. L. (1992). Evolution of the flagellar waveform of ram spermatozoa in relation to the degree of epididymal maturation. Cytoskeleton, 23(1), 8-18.

Comizzoli, P., Songsasen, N., & Wildt, D. E. (2010). Protecting and extending fertility for females of wild and endangered mammals. In oncofertility, (pp. 87-100). Springer US.

Comizzoli, P., Wildt, D. E., & Pukazhenthi, B. S. (2006). Poor centrosomal function of cat testicular spermatozoa impairs embryo development in vitro after intracytoplasmic sperm injection. Biology of reproduction, 75(2), 252-260.

Comizzoli, P., & Wildt, D. E. (2012). On the horizon for fertility preservation in domestic and wild carnivores. Reproduction in domestic animals, 47(s6), 261-265.

Comizzoli, P., Wildt, D. E., & Pukazhenthi, B. S. (2004). Effect of 1, 2-propanediol versus 1, 2-ethanediol on subsequent oocyte maturation, spindle integrity, fertilization, and embryo development in vitro in the domestic cat. Biology of reproduction, 71(2), 598-604.

Comizzoli, P., Wildt, D. E., & Pukazhenthi, B. S. (2008). Impact of anisosmotic conditions on structural and functional integrity of cumulus–oocyte complexes at the germinal vesicle stage in the domestic cat. Molecular reproduction and development, 75(2), 345-354.

Cooper, T. G. (1998). Interactions between epididymal secretions and spermatozoa. Journal of reproduction and fertility. Supplement, 53, 119-136.

Crozet, N., Dahirel, M., & Chesne, P. (2000). Centrosome inheritance in sheep zygotes: centrioles are contributed by the sperm. Microscopy research and technique, 49(5), 445-450.

Dacheux, J. L., & Dacheux, F. (2002). Protein secretion in the epididymis. The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Publishers, 151-168.

Dacheux, J.L., J.L. Gatti, and F.o. Dacheux. (2003). Contribution of epididymal secretory proteins for spermatozoa maturation. Microscopy research and technique, 61(1): p. 7-17.

Dacheux, J.-L. and F.o. Dacheux. (2014). New insights into epididymal function in relation to sperm maturation. Reproduction, 147(2): p. R27-R42.

Delgehyr, N., Sillibourne, J., & Bornens, M. (2005). Microtubule nucleation and anchoring at the centrosome are independent processes linked by ninein function. Journal of cell science, 118(8), 1565-1575.

D'Amours, O., Bordeleau, L. J., Frenette, G., Blondin, P., Leclerc, P., & Sullivan, R. (2012). Binder of sperm 1 and epididymal sperm binding protein 1 are associated with different bull sperm subpopulations. Reproduction, 143(6), 759-771.

Eickhoff, R., Wilhelm, B., Renneberg, H., Wennemuth, G., Bacher, M., Linder, D., Bucala, R., Seitz, J. and Meinhardt, A. (2001). Purification and characterization of macrophage migration inhibitory factor as a secretory protein from rat epididymis: evidences for alternative release and transfer to spermatozoa. Molecular medicine, 7(1), 27.

Elliott, G. D., Lee, P. C., Paramore, E., Van Vorst, M., & Comizzoli, P. (2015). Resilience of oocyte germinal vesicles to microwave-assisted drying in the domestic cat model. Biopreservation and biobanking, 13(3), 164-171.

Fawcett, D. W. (1975). The mammalian spermatozoon. Developmental Biology, 44(2): 394-436.

Fawcett, D. W. and S. Ito. (1965). The fine structure of bat spermatozoa. American journal of anatomy, 116(3): 567-609.

Fornes, M., A. Barbieri, and J. Cavicchia. (1995). Morphological and enzymatic study of membrane-bound vesicles from the lumen of the rat epididymis. Andrologia, 27(1): p. 1-5.

Frenette, G., Lessard, C., Madore, E., Fortier, M. A., & Sullivan, R. (2003). Aldose reductase and macrophage migration inhibitory factor are associated with epididymosomes and spermatozoa in the bovine epididymis. Biology of reproduction, 69(5), 1586-1592.

Fouchecourt, S., Sonia Métayer, S., Locatelli, A., Dacheux F., Dacheux, J. (2000). Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins. Biology of reproduction, 62(6): p. 1790-1803.

Frenette, G., C. Lessard, and R. Sullivan. (2002). Selected proteins of “prostasome-like particles” from epididymal cauda fluid are transferred to epididymal caput spermatozoa in bull. Biology of reproduction, 67(1): p. 308-313.

Frenette, G. and R. Sullivan. (2001). Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. Molecular reproduction and development, 59(1): p. 115-121.

Gaglio, T., Saredi, A., & Compton, D. A. (1995). NuMA is required for the organization of microtubules into aster-like mitotic arrays. The Journal of cell biology, 131(3), 693-708.

Gagnon, C. (1999). The male gamete: From basic to clinical applications.

Gatti, J. L., Métayer, S., Belghazi, M., Dacheux, F., & Dacheux, J. L. (2005). Identification, proteomic profiling, and origin of ram epididymal fluid exosome-like vesicles. Biology of reproduction, 72(6), 1452-1465.

Girouard, J., Frenette, G., & Sullivan, R. (2011). Comparative proteome and lipid profiles of bovine epididymosomes collected in the intraluminal compartment of the caput and cauda epididymidis. International journal of andrology, 34(5pt2).

Gómez, M. C., Pope, E., Harris, R., Mikota, S., & Dresser, B. L. (2003). Development of in vitro matured, in vitro fertilized domestic cat embryos following cryopreservation, culture and transfer. Theriogenology, 60(2), 239-251.

Goto, M., O'brien, D. A., & Eddy, E. M. (2010). Speriolin is a novel human and mouse sperm centrosome protein. Human reproduction, 25(8), 1884-1894.

Gould, M. C. and J. L. Stephano. (1999). MAP Kinase, Meiosis, and Sperm Centrosome Suppression in Urechis caupo. Developmental biology, 216(1): 348-358.

Hermo, L. and D. Jacks. (2002). Nature's ingenuity: bypassing the classical secretory route via apocrine secretion. Molecular reproduction and development, 63(3): p. 394-410.

Hinchcliffe, E. H., Miller, F. J., Cham, M., Khodjakov, A., & Sluder, G. (2001). Requirement of a centrosomal activity for cell cycle progression through G1 into S phase. Science, 291(5508), 1547-1550.

Hoyer-Fender, S. (2012). Centrosomes in fertilization, early embryonic development, stem cell division, and cancer.

Hüber, H.-F. (2007). Alternative splicing of exon 3b gives rise to ODF2 and Cenexin. Cytogenetic and genome research, 119(1-2): 68-73.

Ishikawa H, K. A. T. S. T. S. (2005). Odf2-deficient mother centrioles lack distal/subdistal appendages and the ability to generate primary cilia. Nature cell biology, 7(5): 517-24.

IUCN Red List of Threatened Species. Version 2017.1. <http://www.iucnredlist.org>

Jewgenow, K., Penfold, L. M., Meyer, H. H. D., & Wildt, D. E. (1998). Viability of small preantral ovarian follicles from domestic cats after cryoprotectant exposure and cryopreservation. Journal of reproduction and fertility, 112(1), 39-47.

Johnston, L. A., O'brien, S. J., & Wildt, D. E. (1989). In vitro maturation and fertilization of domestic cat follicular oocytes. Molecular reproduction and development, 24(3), 343-356.

Karja, N. W. K., Otoi, T., Murakami, M., Fahrudin, M., & Suzuki, T. (2002). In vitro maturation, fertilization and development of domestic cat oocytes recovered from ovaries collected at three stages of the reproductive cycle. Theriogenology, 57(9), 2289-2298.

Khodjakov, A., Cole, R. W., Oakley, B. R., & Rieder, C. L. (2000). Centrosome-independent mitotic spindle formation in vertebrates. Current biology, 10(2), 59-67.

Kim, Y., Selvaraj, V., Pukazhenthi, B., & Travis, A. J. (2007). Effect of donor age on success of spermatogenesis in feline testis xenografts. Reproduction, Fertility and development, 19(7), 869-876.

Kirchhoff, C., & Hale, G. (1996). Cell-to-cell transfer of glycosylphosphatidylinositol-anchored membrane proteins during sperm maturation. MHR: Basic science of reproductive medicine, 2(3), 177-184.

Kitiyanant, Y., Saikhun, J., & Pavasuthipaisit, K. (2003). Somatic cell nuclear transfer in domestic cat oocytes treated with IGF-I for in vitro maturation. Theriogenology, 59(8), 1775-1786.

Lange, B. M. and K. Gull. (1995). A molecular marker for centriole maturation in the mammalian cell cycle. The journal of cell biology, 130(4): 919-927.

Légaré, C., Bérubé, B., Boué, F., Lefièvre, L., Morales, C. R., El-Alfy, M., & Sullivan, R. (1999). Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein. Molecular reproduction and development, 52(2), 225-233.

Lengwinat, T., & Blottner, S. (1994). In vitro fertilization of follicular oocytes of domestic cat using fresh and cryopreserved epididymal spermatozoa. Animal reproduction science, 35(3-4), 291-301.

Lingle, W. L., Lutz, W. H., Ingle, J. N., Maihle, N. J., & Salisbury, J. L. (1998). Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. Proceedings of the National Academy of Sciences, 95(6), 2950-2955.

Luvoni, G. C., & Pellizzari, P. (2000). Embryo development in vitro of cat oocytes cryopreserved at different maturation stages. Theriogenology, 53(8), 1529-1540.

Luvoni, G. C., Pellizzari, P., & Battocchio, M. (1997). Effects of slow and ultrarapid freezing on morphology and resumption of meiosis in immature cat oocytes. Journal of reproduction and fertility. Supplement, 51, 93-98.

Martin-DeLeon, P. A. (2006). Epididymal SPAM1 and its impact on sperm function. Molecular and cellular endocrinology, 250(1), 114-121.

Manandhar, G., Schatten, H., & Sutovsky, P. (2005). Centrosome reduction during gametogenesis and its significance. Biology of reproduction, 72(1), 2-13.

McNally, K. L., Fabritius, A. S., Ellefson, M. L., Flynn, J. R., Milan, J. A., & McNally, F. J. (2012). Kinesin-1 prevents capture of the oocyte meiotic spindle by the sperm aster. Developmental cell, 22(4), 788-798.

Morales, A. and J.C. Cavicchia. (1991). Release of cytoplasmic apical protrusions from principal cells of the cat epididymis, an electron microscopic study. Tissue and cell, 23(4): p. 505-513.

Mouttham, L., & Comizzoli, P. (2016). The preservation of vital functions in cat ovarian tissues during vitrification depends more on the temperature of the cryoprotectant exposure than on the sucrose supplementation. Cryobiology, 73(2), 187-195.

Murakami, M. K., Otoi, T., Karja, N. W. K., Wongsrikeao, P., Agung, B., & Suzuki, T. (2004). Blastocysts derived from in vitro-fertilized cat oocytes after vitrification and dilution with sucrose. Cryobiology, 48(3), 341-348.

Navara, C., N. First, and G. Schatten. (1996). Phenotypic variations among paternal centrosomes expressed within the zygote as disparate microtubule lengths and sperm aster organization: correlations between centrosome activity and developmental success. Proceedings of the National Academy of Sciences, 93(11): p. 5384-5388.

Neubauer, K., Jewgenow, K., Blottner, S., Wildt, D. E., & Pukazhenthi, B. S. (2004). Quantity rather than quality in teratospermic males: a histomorphometric and flow cytometric evaluation of spermatogenesis in the domestic cat (Felis catus). Biology of reproduction, 71(5), 1517-1524.

Nicander, L., & Malmqvist, M. (1977). Ultrastructural observations suggesting merocrine secretion in the initial segment of the mammalian epididymis. Cell and tissue research, 184(4), 487-490.

Ou, Y. Y., Mack, G. J., Zhang, M., & Rattner, J. B. (2002). CEP110 and ninein are located in a specific domain of the centrosome associated with centrosome maturation. Journal of cell science, 115(9), 1825-1835.

Palermo, G. D., Colombero, L. T., & Rosenwaks, Z. (1997). The human sperm centrosome is responsible for normal syngamy and early embryonic development. Reviews of reproduction, 2(1), 19-27.

Pihan, G. A., Purohit, A., Wallace, J., Knecht, H., Woda, B., Quesenberry, P., & Doxsey, S. J. (1998). Centrosome defects and genetic instability in malignant tumors. Cancer research, 58(17), 3974-3985.

Platz, C. C., Wildt, D. E., & Seager, S. W. J. (1978). Pregnancy in the domestic cat after artificial insemination with previously frozen spermatozoa. Journal of reproduction and fertility, 52(2), 279-282.

Pope, C. E., Turner, J. L., Quatman, S. P., & Dresser, B. L. (1991). Semen storage in the domestic felid: a comparison of cryopreservation methods and storage temperatures. Biology of reproduction, 44(Supplement 1), 117.

Pukazhenthi, B., Comizzoli, P., Travis, A. J., & Wildt, D. E. (2005). Applications of emerging technologies to the study and conservation of threatened and endangered species. Reproduction, fertility and development, 18(2), 77-90.

Pukazhenthi, B. S., Neubauer, K., Jewgenow, K., Howard, J., & Wildt, D. E. (2006). The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology, 66(1), 112-121.

Pukazhenthi, B., Pelican, K., Wildt, D., & Howard, J. (1999). Sensitivity of domestic cat (Felis catus) sperm from normospermic versus teratospermic donors to cold-induced acrosomal damage. Biology of reproduction, 61(1), 135-141.

Pukazhenthi, B., Spindler, R., Wildt, D., Bush, L. M., & Howard, J. (2002). Osmotic properties of spermatozoa from felids producing different proportions of pleiomorphisms: influence of adding and removing cryoprotectant. Cryobiology, 44(3), 288-300.

Pukazhenthi, B. S., Wildt, D. E., & Howard, J. G. (2000). The phenomenon and significance of teratospermia in felids. Journal of reproduction and fertility. Supplement, 57, 423-433.

Rawe, V. Y., Terada, Y., Nakamura, S., Chillik, C. F., Olmedo, S. B., & Chemes, H. E. (2002). A pathology of the sperm centriole responsible for

defective sperm aster formation, syngamy and cleavage. Human reproduction, 17(9), 2344-2349.

Rejraji, H., Vernet, P., & Drevet, J. R. (2002). GPX5 is present in the mouse caput and cauda epididymidis lumen at three different locations. Molecular reproduction and development, 63(1), 96-103.

Robbins, E., Jentzsch, G., & Micali, A. (1968). The centriole cycle in synchronized HeLa cells. The Journal of cell biology, 36(2), 329-339.

Saez, F., Frenette, G., & Sullivan, R. (2003). Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. Journal of andrology, 24(2), 149-154.

Salmon, N. A., Reijo Pera, R. A., & Xu, E. Y. (2006). A gene trap knockout of the abundant sperm tail protein, outer dense fiber 2, results in preimplantation lethality. Genesis, 44(11), 515-522.

Sathananthan, A. H., Tatham, B., Dharmawardena, V., Grills, B., Lewis, I., & Trounson, A. (1997). Inheritance of sperm centrioles and centrosomes in bovine embryos. Archives of andrology, 38(1), 37-48.

Schatten, G. (1994). The Centrosome and Its Mode of Inheritance: The Reduction of the Centrosome during Gametogenesis and Its Restoration during Fertilization. Developmental biology, 165(2): 299-335.

Schatten, H. and Q.-Y. Sun. (2009). The role of centrosomes in mammalian fertilization and its significance for ICSI. Molecular human reproduction, 15(9): 531-538.

Schmehl, M. and E. Graham. (1989). Ultrastructure of the domestic tom cat (Felisdomestica) and tiger (Pantheratigrisaltaica) spermatozoa. Theriogenology, 31(4): 861-874.

Schmidt, T. I., Kleylein-Sohn, J., Westendorf, J., Le Clech, M., Lavoie, S. B., Stierhof, Y. D., & Nigg, E. A. (2009). Control of centriole length by CPAP and CP110. Current biology, 19(12), 1005-1011.

Senger, P.L., Pathways to pregnancy and parturition. 2 ed. 2005: Current Conceptions, Inc., 1615 NE Eastgate Blvd.

Spektor, A., Tsang, W. Y., Khoo, D., & Dynlacht, B. D. (2007). Cep97 and CP110 suppress a cilia assembly program. Cell, 130(4), 678-690.

Spindler, R. E., & Wildt, D. E. (1999). Circannual variations in intraovarian oocyte but not epididymal sperm quality in the domestic cat. Biology of reproduction, 61(1), 188-194.

Snedaker, A. K., Honaramooz, A., & Dobrinski, I. (2004). A game of cat and mouse: xenografting of testis tissue from domestic kittens results in complete cat spermatogenesis in a mouse host. Journal of andrology, 25(6), 926-930.

Stephano, J. L. and M. C. Gould. (2000). MAP kinase, a universal suppressor of sperm centrosomes during meiosis? Developmental biology, 222(2): 420-428.

Sullivan, R. (1999). Interaction between sperm and epididymal secretory proteins. The male gamete: from basic to clinical applications, 130-136.

Sullivan, R., Frenette, G., & Girouard, J. (2007). Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. Asian journal of andrology, 9(4), 483-491.

Swanson, W. F., & Godke, R. A. (1994). Transcervical embryo transfer in the domestic cat. Laboratory animal science (USA).

Syntin, P., Dacheux, F., Druart, X., Gatti, J. L., Okamura, N., & Dacheux, J. L. (1996). Characterization and identification of proteins secreted in the various regions of the adult boar epididymis. Biology of reproduction, 55(5), 956-974.

Szebenyi, G., Hall, B., Yu, R., Hashim, A. I., & Krämer, H. (2007). Hook2 localizes to the centrosome, binds directly to centriolin/CEP110 and contributes to centrosomal function. Traffic, 8(1), 32-46.

“Testis.” 2015; Available from: http://www.britannica.com/EBchecked/topic/588769/testis.

Thimon, V., Koukoui, O., Calvo, E., & Sullivan, R. (2007). Region-specific gene expression profiling along the human epididymis. Molecular human reproduction, 13(10), 691-704.

Tsutsui, T., Tanaka, A., Takagi, Y., Nakagawa, K., Fujimoto, Y., Murai, M., Anzai, M., and Hori, T. (2000). Unilateral intrauterine horn insemination of frozen semen in cats. Journal of veterinary medical science, 62(12), 1247-1251

Tsutsui, T., Wada, M., Anzai, M., and Hori, T. (2003). Artificial insemination with frozen epididymal sperm in cats. Journal of veterinary medical science, 65, no. 3: 397-399.

Turner, R. M. (2005). Moving to the beat: a review of mammalian sperm motility regulation. Reproduction, fertility and development, 18(2), 25-38

Vorobjev, I. A., & YuS, C. (1982). Centrioles in the cell cycle. I. Epithelial cells. The Journal of cell biology, 93(3), 938-949.

Wildt, D. E., Comizzoli, P., Pukazhenthi, B., & Songsasen, N. (2010). Lessons from biodiversity—the value of nontraditional species to advance reproductive science, conservation, and human health. Molecular reproduction and development, 77(5), 397-409.

Wildt, D. E., Ellis, S., & Howard, J. G. (2001). Linkage of reproductive sciences: from 'quick fix' to 'integrated' conservation. Journal of reproduction and fertility. Supplement, 57, 295-307.

Wu, X., & Palazzo, R. E. (1999). Differential regulation of maternal vs. paternal centrosomes. Proceedings of the National Academy of Sciences, 96(4), 1397-1402.

Yin, X., Lee, Y., Lee, H., Kim, N., Kim, L., Shin, H., & Kong, I. (2006). In vitro production and initiation of pregnancies in inter-genus nuclear transfer embryos derived from leopard cat (Prionailurus bengalensis) nuclei fused with

domestic cat (Felis silverstris catus) enucleated oocytes. Theriogenology, 66(2), 275-282.

Zambelli, D., Caneppele, B., Castagnetti, C., & Belluzzi, S. (2002). Cryopreservation of cat semen in straws: comparison of five different freezing rates. Reproduction in domestic animals, 37(5), 310-313.

Zamboni, L. and M. Stefanini. (1971). The fine structure of the neck of mammalian spermatozoa. The Anatomical Record, 169(2): 155-172.

Zheng, Y., Wong, M. L., Alberts, B., & Mitchison, T. (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. Nature, 378(6557), 578.

Chapter 2: Deciphering the mechanisms involving cenexin, ninein, and centriolin in sperm maturation during epididymal transit in the domestic cat.

Abstract

The sperm centrosome is an essential organelle with a key role in organizing the sperm aster for proper syngamy and formation of the first mitotic spindle. The sperm cell acquires the functional capability during epididymal transit by incorporation of key factors. The objective of the study was to identify key maturation proteins, such as ninein and centriolin, as well as cenexin- a scaffold protein that serves to bind ninein and centriolin. Epididymal samples were dissected from 17 adult cat testes (>1 year-old, *Felis catus*) and spermatozoa extracted from the different regions, including rete testis, caput, corpus, cauda, and vas deferens. Tissue samples and sperm cells were fixed separately in 4% paraformaldehyde before immunostaining with anti-cenexin, ninein, or centriolin antibodies. Results showed that the proportion of sperm cells with cenexin localized at the centrosome progressively increased along the tract with the lowest percentage of stained cells in the testis (mean= 45%) and highest in the cauda (mean= 81%). Although not significant, the intensity of cenexin immunofluorescence in positive cells increased two-fold from the testis to vas deferens. There was no significant difference in the proportion of sperm labeled with centriolin or ninein (ranges of 21-26%, 33-48% between segments,

respectively) or the intensity (±58%, ±63% change as compared to testis between segments, respectively). Cenexin may serve as a scaffold protein for centriolin and ninein, as the vast majority of spermatozoa only displayed co-localization of these proteins when cenexin was present (mean= 85% and 91% co-localization, respectively). In summary, these results could be applied to future efforts to create treatments to rescue the impaired centrosome of an infertile male, with particular potential for wild felid conservation.

Introduction

The sperm centrosome is an essential organelle playing a key role in the fertilization process just after penetration into the oocyte. It serves to organize the sperm aster which is required for proper syngamy and formation of the first mitotic spindle. The centrosome is located at the base of the sperm head and, following oocyte penetration, will migrate to the center of the oocyte to produce a mass array of microtubules, collectively referred to as the sperm aster. These microtubules will bind with the male and female pronuclei and physically draw them together for pronuclear fusion (Schatten, 1994; Navara et al., 1996). Our laboratory previously demonstrated the importance of centrosome maturation by injecting domestic cat oocytes with either mature, ejaculated spermatozoa or immature spermatozoa collected from the testis segment (Comizzoli et al., 2006). Oocytes injected with mature spermatozoa showed formation of a large, competent sperm aster. Conversely, injection of immature spermatozoa resulted in small asters and significantly increased time for the onset of first

cleavage and embryonic death. These differences point to key maturational processes that occur specifically for the centrosome at the time that the whole sperm cell is undergoing maturation within the male reproductive tract. Improper maturation has also been associated with infertility in species such as human and bovine (Rawe et al., 2002; Navara et al., 2006) in which the injection of sperm collected from infertile individuals resulted in diminished aster production and pronuclear fusion.

While the importance of the sperm centrosome has been identified in many species the underlying physiological processes that aid in its maturation are poorly understood. The proteins, ninein, and centriolin have been reported in the maturation and function of somatic cell centrosomes (Delghyr et al., 2005, Ishikawa et al., 2005). Specifically, ninein serves in the nucleation and anchoring of microtubules, and centriolin aids in the formation of the centrosome during early cellular development. Additionally, cenexin has been reported to act as a scaffold protein to allow further binding of other proteins, including ninein and centriolin, to the distal appendages of the mature centriole (Ishikawa et al., 2005). While these proteins and their functions have been identified in somatic cells, their role in the sperm centrosome's maturation and function has yet to be investigated in any species. Therefore, the aim of this study was to identify whether certain maturational proteins (cenexin, ninein,

and centriolin) are incorporated by the centrosome as the sperm cell completes its epididymal transit.

The identification of these proteins and characterization of their secretion patterns will provide critical insights for the application of an *in vitro* culture system designed to rescue centrosome function of an individual that exhibits this type of infertility. This could be especially beneficial for the conservation efforts of endangered species in which it is imperative that spermatozoa from genetically valuable individuals are able to be used for fertilization.

Materials and Methods

Sample collection and preparation

All reagents were attained from Sigma Aldrich (St. Louis, MO) unless noted otherwise. Testes from 17 adult (>1 year) domestic cats were harvested during routine orchiectomy and donated by local veterinary clinics. The tracts were transported and stored in phosphate buffered saline (Gibco, DPBS) at 4°C until processing. Spermatozoa were then extracted from the different regions (rete testis, caput, corpus, cauda, and vas deferens) by slicing with a scalpel blade in complete Hepes-Ham medium F10 (25 mM Hepes, 1.0 mM pyruvate, 2.0 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 5% fetal bovine serum) at 37°C and processed separately. Cell suspensions from

each sample were centrifuged (300 x g, 8 min) and suspended in fresh Hepes-Ham F10 medium.

Immunofluorescent Microscopy of Sperm Cells

Following sample collection, 20µL of each sperm suspension was smeared on a glass slide and fixed with 4% paraformaldehyde (1hr, room temp) followed by permeabilization with 0.1% Triton X-100 in PBS (PBS-T) for 3min. The cells were blocked in 5% bovine serum albumin in PBS (1hr, room temperature) and incubated with anti-cenexin (1/100, Millipore #MABT154), ninein (1/100, Abcam #ab4447), or centriolin antibodies (1/200, Abcam #ab99337) overnight at 4°C within a humidified chamber. After washings (5 min each) in PBS twice and PBS-T once, samples were incubated with secondary antibodies labeled with a fluorescent probe for 1hr at 37°C (cenexin= 1/100, Sigma #F0257; ninein and centriolin= 1/100, Sigma #SAB3700859). Sperm chromatin was then stained with Hoechst 33258 (1/100, Sigma #94403) before observation under a microscope fitted with epifluorescence (Olympus BX 41). Following antigen retrieval using a citric acid wash buffer (0.2% citric acid w/v, 0.1% w/v ethylenediaminetetraacetic acid, 0.25% Triton X-100 20 min at 95 °C), immunofluorescent labeling of tissue samples was conducted in the same manner as previously described.

The proportion of sperm cells that exhibited positive detection at the location of the centrosome (Figure 2.1) was recorded for each region of the reproductive tract, two hundred cells were analyzed per segment of each individual male (cenexin= 9 males, ninein= 5 males, and centriolin= 4 males).

The proportion of sperm cells that exhibited co-localization of cenexin with either ninein or centriolin was also recorded for each segment, analyzing 100 cells total per segment of each individual (n= 4 males for each protein). Images of positively labeled sperm (30 image per segment of each individual, n= 4 males for each protein) were captured using Spot Basic 5.1 software (Diagnostics Instruments). The pixel intensity of immunofluorescence (mean grey value) was then analyzed using ImageJ version 1.47 software to indicate the relative amount of protein bound with the centrosome. To calibrate the intensities between different sperm cells, the area measured was adjusted according to the size of each cell's centrosome and the intensity expressed as the averaged mean grey value recorded per pixel. Following these measurements, variations between segments of each individual was also calibrated by subtracting the averaged background intensity of the negative segment control sample (sample labeled with the secondary fluorescent antibody only, primary antibody omitted) and the background subtracted from each positively labeled cell to produce relative intensity. The change in relative intensity of the caput, corpus, cauda, and vas deferens segments was then analyzed in comparison to the testis segment.

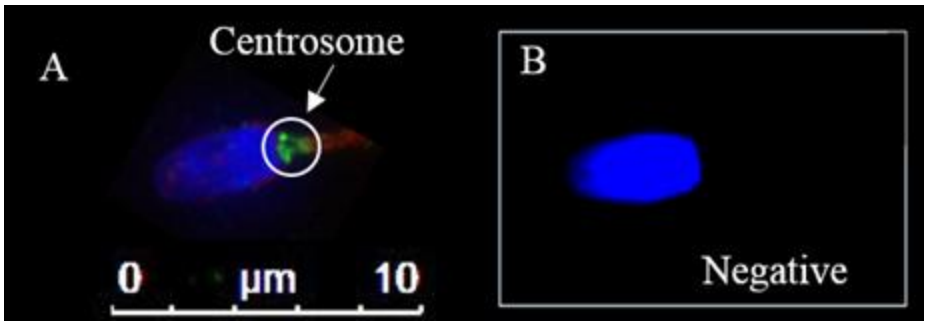


Figure 2.1. Example of protein localization (cenexin) at the centrosome within sperm cells, collected from the cauda segment. A= FITC, cenexin and Texas Red, centriolin; B= negative control of cenexin stain.

Experimental Design and Statistical Analyses

On each experimental day (two males, n= 4-9 total males per experiment), testes were collected from multiple individuals and the spermatozoa isolated from the different regions (rete testis, caput, corpus, cauda, and vas deferens), proteins of interest were then stained and analyzed via immunofluorescent microscopy. Following these preparations, the proportion of positively labeled cells and the intensity of staining at the location of the centrosome was quantified. Statistical analyses were conducted via GraphPad Prism software version 6 (GraphPad Software, Inc). The proportion of positively stained sperm collected from each segment and the corresponding intensity of stain were analyzed using repeated measures with results further compared via protected Tukey's test. The proportion of spermatozoa that displayed co-localization of ninein or centriolin with cenexin was compared against the proportion of sperm which did not exhibit co-localization using a paired Student's t-test within each segment, blocking for individual variation.

Results

The proportion of sperm cells with cenexin localized at the centrosome progressively increased along the tract with the lowest percentage of stained cells in the testis (45%) and highest in the cauda (81%, $P<0.01$; Figure 2.2). Although not significant, the intensity of cenexin immunofluorescence in

positive cells also tended to increase from the testis to vas deferens (increased twofold, $P>0.05$; Figure 2.3).

There was no significant difference ($P>0.05$) in the proportion of sperm labeled with centriolin (range, 21 to 26%) or ninein (range, 33 to 48%) across segments (Figure 2.2). The intensity of the labelling did not differ across segments ($P>0.05$) compared to testis for centriolin (range, -50% to +7% change) and ninein (range, -40 to +23% change; Figure 2.3). The vast majority of spermatozoa only displayed co-localization of ninein or centriolin when cenexin was also present (centriolin= range 67 to 88%, mean= 85% co-localization, $P<0.01$; and ninein= range 90 to 100%, mean= 91% co-localization, $P<0.01$; across segments).

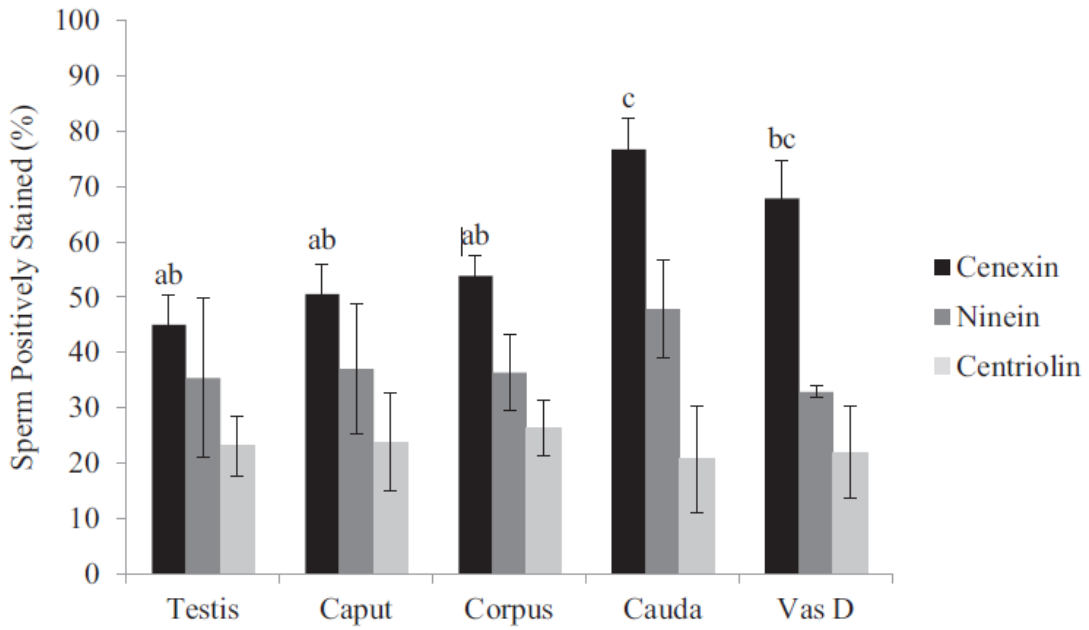


Figure 2.2. Proportion of sperm positively stained for cenexin, ninein and centriolin at the centrosome across the epididymal segments (mean ± SEM, cenexin = 9 males, ninein = 5 males, centriolin = 4 males). Bars with different letters indicate significant differences in the proportion of positively stained sperm for cenexin between the consecutive segments ($P < 0.01$).

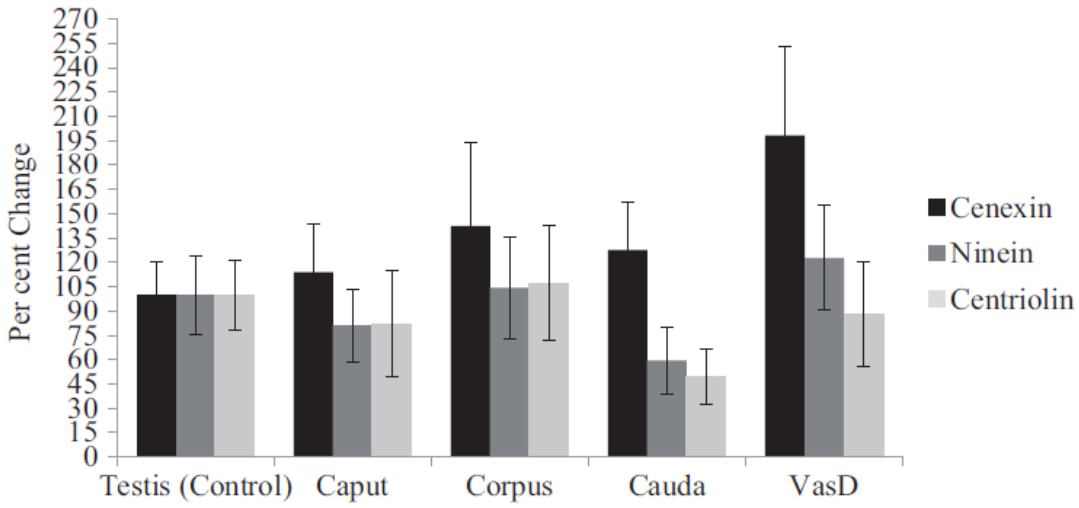


Figure 2.3. Relative immunofluorescence intensity (relative to the fluorescence in the testis) of cenexin, ninein and centriolin across the different epididymal segments (mean ± SEM, n = 4 males for each protein, P> 0.05).

Discussion

For the first time, three maturational proteins were detected and co-localized in the cat sperm centrosome. Cenexin was detected in a significantly increasing proportion of the sperm population with the intensity also displaying an increasing trend. Additionally, a large proportion of sperm cells also showed co-localization of ninein or centriolin with cenexin. This is indicative of its potential role as a scaffold for other proteins as it has been observed in somatic cells (Ishikawa et al., 2005). Binding with cenexin prior to fertilization could allow the centrosome to acquire a greater amount of other maturational proteins, thus enabling it to better prepare for its function post-fertilization.

No significant differences were detected in the localization or staining intensity of ninein and centriolin. It is possible that these proteins are also recruited from the oocyte's cytoplasm to the centrosome following fertilization as has been detected with other essential proteins such as γ-tubulin in Xenopus (Stearns and Kirschner, 1994) and centrosomin in Drosophila (Dix and Raff, 2007). Thus, while these proteins may begin to bind with the centrosome during the sperm's maturation, additional association may not occur until recruitment within the oocyte.

Ou and coworkers (2002) observed an association between the presence of ninein and centriolin with microtubule assembly in PtK2 cells. Injecting anti-centriolin or anti-ninein antibodies interfered with the centrosome's ability to organize the microtubules, indicating their requirement for the formation

process. It is therefore possible that these proteins may also serve a role in the production of the sperm aster. If so, then the observed progressive binding of cenexin to the sperm centrosome suggests a potential role in the accumulation of these proteins during epididymal sperm transit and post-fertilization.

Together, our data provide evidence for the maturational role of cenexin, and the potential role of ninein, and centriolin in the sperm centrosome's function following oocyte penetration. Analyses to characterize the locations of protein secretion throughout the reproductive tract are currently underway. By furthering the understanding of these physiological processes, we will improve the use of assisted reproductive techniques for *in vitro* sperm maturation. While impaired centrosome development has been identified as a source of infertility in certain species such as the human and bovine (Rawe et al., 2002; Navara et al., 2006), the ability to rescue the function of this organelle has yet to be accomplished. Thus, the results of this study are applicable to the future development of an *in vitro* culture system for this type of infertility. This technology can be applied to human infertility treatments, as well as the rescue of sperm function in genetically valuable individuals such as in an endangered felid species.

Conclusion

This study has identified three proteins- cenexin, ninein, and centriolin, that are associated with the sperm centrosome in the domestic cat, and has

characterized the timing of incorporation throughout the sperm epididymal transit. These data are among the first to demonstrate protein integration and its relation to the sperm centrosome maturation in mammalian species. The information gained from this study is critical to better understand the development of the centrosome and provides valuable insight critical to improve the treatment of centrosomal-related infertility, and development of assisted reproductive technologies.

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References

Comizzoli P., Wildt D. E., and Pukazhenthi B.S. (2006). Poor centrosomal function of cat testicular spermatozoa impairs embryo development in vitro after intracytoplasmic sperm injection. Biology of reproduction, 75 (2), 252-260.

Delgehyr N., Sillibourne J., and Bornens M. (2005). Microtubule nucleation and anchoring at the centrosome are independent processes linked by ninein function. Journal of cell science, 118 (8), 1565-1575.

Dix C.I., and Raff J.W. (2007). Drosophila Spd-2 recruits PCM to the sperm centriole, but is dispensable for centriole duplication. Current biology, 17 (20), 1759-1764.

Ishikawa H., Kubo A., Tsukita T., Tsukita S. (2005). Odf2-deficient mother centrioles lack distal/subdistal appendages and the ability to generate primary cilia. Nature cell biology, 7 (5), 517-24.

Navara C., First N., and Schatten G. (1996). Phenotypic variations among paternal centrosomes expressed within the zygote as disparate microtubule lengths and sperm aster organization: correlations between centrosome activity and developmental success. Proceedings of the National Academy of Sciences, 93(11), 5384-5388.

Ou, Y.Y., Mack G.J., Zhang M., and Rattner J.B. (2002). CEP110 and ninein are located in a specific domain of the centrosome associated with centrosome maturation. Journal of cell science, 115(9), 1825-1835.

Rawe V.Y., Terada Y., Nakamura C.F. Chillik1 S., Brugo O., and Chemes H.E. (2002). A pathology of the sperm centriole responsible for defective sperm aster formation, syngamy and cleavage. Human reproduction, 17(9), 2344-2349.

Schatten, G. (1994). The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. Developmental biology, 165(2), 299-335.

Stearns T., and Kirschner M. (1994). In vitro reconstitution of centrosome assembly and function: the central role of γ-tubulin. Cell, 76.4, 623-637.

Chapter 3: Key factors enhancing sperm fertilizing ability are transferred from the epididymis to the spermatozoa via epididymosomes in the domestic cat model.

Abstract

Spermatozoa undergo critical changes in structure and function during the epididymal transit. Previous studies in the domestic cat demonstrated that incidence of cenexin- a key protein involved in the centrosomal maturation – progressively increases in sperm cells from caput to cauda epididymis. Study objectives were to (1) characterize mechanisms involved in transferring key factors – using the cenexin as a marker – between the epididymis and maturing sperm cells and (2) demonstrate the impact of such mechanisms on the acquisition of functional properties by spermatozoa. Epididymides were dissected from adult cat testes to assess the presence and localization of cenexin in testicular tissues and each epididymal segment (caput, corpus, and cauda) via immunofluorescence, western blot and mass spectrometry. Results showed that tissues, luminal fluid, and isolated epididymosomes from each segment contained cenexin. Co-incubation of immature sperm cells for 3 h with luminal fluid or epididymosomes followed by immunostaining revealed a significant increase in percentages of sperm cells containing cenexin. Additionally, epididymosome co-incubation with immature spermatozoa resulted in sustained motility compared to untreated spermatozoa which displayed a decline over the incubation period. Analyses of acrosome integrity revealed no

significant effects following incubation. Taken together, these results point to a critical role for epididymosomes in epididymal sperm maturation and as such, could be a key modulator for enhancing or suppressing male fertility.

Introduction

Understanding the physiological processes leading to structurally and functionally mature spermatozoa is critical for overcoming common problems with infertility or developing efficient contraception strategies. Specifically, sperm maturation in the epididymis is fundamental for the cell to acquire the ability to fertilize an oocyte. Even though thorough reports exist on the sperm maturation process in several species (Dacheux J-L et al., 2012), many mechanisms remain to be deciphered regarding the epididymal sperm maturation in the domestic cat (Axnér, 2006)– a critical model for biomedical studies and wild felids conservation. Besides important morphological changes, sperm maturation is associated with the integration of specific factors including peptides and microRNA (Dacheux and Dacheu, 2014; Sullivan R, Saez, 2013). Classical methods of protein secretion by the epithelium of the epididymis involve the merocrine pathway in which proteins contain signal sequences and are secreted individually (Nicander and Malmqvis, 1977; Dacheux and Dacheux, 2014). Once secreted into the luminal fluid the proteins then bind with the sperm surface to be integrated by the cell (Cooper, 1998; Sullivan et al., 2007; Kirchhoff and Hale, 1996). Conversely, other secreted proteins without a signal sequence are secreted within small vesicles termed

'epididymosomes'. These small vesicles are secreted from the apical pole of the epididymal epithelial cells in an apocrine manner (Hermo and Jacks, 2002). This type of protein secretion and delivery to the maturing sperm cells via small vesicles have been demonstrated in other species, including the rat, hamster, cow, and human (Fornés et al., 1995; Légaré et al., 1999; Frenette and Sullivan, 2001; Thimon, 2007). Interestingly, epididymosomes have been isolated and observed in domestic cat, but their role has not been demonstrated (Morales and Cavicchia, 1991).

Although previous studies have assessed the content of epididymosomes (microRNA, peptides, proteins, and other factors; Sullivan and Saez, 2013) much remains unclear about their impact on sperm acquisition of fertilizing ability and motility. Previous studies suggested that the epididymal vesicles likely promote sperm motility as they contribute to significant incorporation of multiple factors that modulate motility including aldose reductase, sorbitol dehydrogenase, and macrophage migration inhibitory factor (Sullivan et al., 2007). Using the domestic cat model, we already have determined that key proteins, such as the cenexin are supplied to the spermatozoa throughout the epididymal transit (Rowlison et al., 2017). A critical aspect is the centrosomal maturation allowing the sperm cell to form a large sperm aster after penetration into the oocytes, thus allowing successful embryo development. This role of the centrosome has been demonstrated in the cow (Navara et al., 1996) and in the domestic cat (Comizzoli et al., 2006). However, the influence of epididymosomes on the acquisition of sperm motility

has not been demonstrated. The objectives of the study were to (1) characterize the transfer of key factors – using the cenexin as a marker – between the epididymis and the maturing sperm cells via exposure to isolated luminal fluid and epididymosomes and (2) demonstrate the impact of exposure on the acquisition of functional properties by the spermatozoa.

Materials and Methods

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Sample collection and preparation

Testes from adult (>1 year) domestic cats were harvested during routine orchiectomy and donated by local veterinary clinics. Tracts were transported and stored in phosphate buffered saline (PBS) at 4°C until processing. Testicular and epididymal tissues were dissected in PBS using a scalpel blade and isolated according to the different regions (rete testis, caput, corpus, and cauda epididymis). Part of the epididymal tissues were sliced with a scalpel blade to recover the luminal fluids and release sperm cells that were recovered after centrifugation at 300 x g for 8 min in PBS medium. Cell debris was discarded from the supernatant by a series of centrifugations at 700 x g for 10 min and 3,000 x g for 10 min at room temperature. The epididymosome fraction was isolated from the remaining luminal fluid by ultracentrifugation at 100,000 x g for 2 h at 4°C and re-suspended in fresh PBS. Aliquots of both

luminal fluid and epididymosome samples were stored at -20°C until further processing. Pellets were further assessed via electron microscopy to confirm isolation of the vesicles without tissue or sperm cell contamination.

Electron microscopy

Pellets obtained after ultracentrifugation were stained with uranyl acetate for 1 min and observations performed using a transmission electron microscope (Zeiss 10 CA Transmission Electron Microscope) at the University of Maryland College Park Laboratory for Biological Ultrastructure, USA.

Tissue processing and immunostaining

Tissue samples were fixed with 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned at a thickness of 5 μm. Samples then underwent 10-min antigen retrieval at 95°C (10mM citric acid and 3mM ethylenediaminetetraacetic acid supplemented with 1% Triton-X), and permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 3 min. The non-specific antigenic sites were blocked in 5% bovine serum albumin in PBS (1hr, room temp) and samples incubated with anti-cenexin (1:100, Millipore, Massachusetts, USA) antibodies overnight at 4°C in a humidified chamber. After washing (5 min each) in PBS twice and PBS-T once, samples were incubated with secondary antibodies labeled with a fluorescent probe for 1hr at 37°C (goat anti-mouse 1:100) before observation under a microscope fitted with epifluorescence (Olympus BX 41). As a negative control samples were labeled with the secondary fluorescent antibody, omitting the primary antibody.

Western Blot analysis and mass spectrometry confirmation

Tissue samples from each segment were isolated and homogenized in Tween-20 lysis buffer (150mM sodium chloride, 50mM Trizma Base, 1% Tween-20) and centrifuged at 14,000 x g for 15 minutes at room temperature for analyses via western blot. Samples were diluted with SDS loading buffer (Boston Bioproducts) and incubated at 95°C for 10 minutes. Samples were then separated by one-dimensional electrophoresis (BioRad 4–15% Mini-PROTEAN TGX Gel) with tissue, epididymosome and luminal fluid samples separated based on equivalent quantity of proteins (30µg total protein per lane as determined by BioRad assay kit using bovine serum albumin as standard). Proteins were transferred to a nitrocellulose membrane (BioRad) and blocked for 1 h at room temperature in 1X tris-buffered saline (154mM Trizma HCl, 1M sodium chloride supplemented with 1% Tween-20 and 7% skim milk powder). Membranes were then incubated overnight at 4°C with anti-cenexin (1:1000, Millipore), and 1 h at room temperature with goat anti-mouse secondary coupled with horseradish peroxidase (1:2000, Sigma) in 1X tris-buffered saline supplemented with 1% Tween-20 and 5% skim milk powder. Membranes were then treated with Clarity Western ECL substrate (BioRad) and imaged with ChemiDoc XRS imaging system (Bio-Rad). Results were also confirmed by gel digest analysis via liquid chromatography-tandem mass spectrometry (LC-MS/MS). These analyses were completed with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive at MS Bioworks, USA.

Co-incubation assays

Epididymosome and luminal fluid samples were diluted to a concentration of 4µg total protein/ µL as determined by BioRad assay kit using bovine serum albumin as standard. Immature testicular spermatozoa (8×10^6 sperm/mL) were then co-incubated for 3 h at 38°C with epididymosome and luminal fluid samples, or in culture medium as control. Following incubation, 20 µL of each sperm suspension was smeared on a glass slide and fixed with 4% paraformaldehyde (1hr, room temp) and further prepared following the same procedures detailed above for the immunofluorescent staining of epididymal tissue. Controls included testicular spermatozoa incubated in the base medium or testicular and cauda spermatozoa fixed without any treatment. Negative control treatments were also included in which the primary antibody was omitted and samples were labeled with the secondary fluorescent antibody only.

Proportion of sperm cells that exhibited positive staining at the location of the centrosome were recorded for each treatment, analyzing 200 cells total per treatment of each individual male. Images of positively labeled sperm (30 images per treatment of each individual) were captured using Spot Basic 5.1 software (Diagnostics Instruments). The pixel intensity of immunofluorescence (mean grey value) was then analyzed using ImageJ version 1.47 software to indicate the relative amount of protein bound with the centrosome. To calibrate the intensities between different sperm cells, the area measured was adjusted according to the size of each cell's centrosome and the intensity expressed as the averaged mean grey value recorded per pixel.

Following these measurements, variations between treatments of each individual was also calibrated by averaging the background intensity of the negative control sample and the value subtracted from each positively labeled cell. The change in relative intensity of each treatment was then analyzed in comparison to the testicular spermatozoa treatment incubated in base medium.

Motility and acrosome integrity assays

Epididymosome samples were diluted to a concentration of 4μg total protein/ μL with Hepes-Ham medium F10 (25 mM Hepes, 1.0 mM pyruvate, 2.0 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 5% fetal calf serum) as determined by BioRad assay kit using bovine serum albumin as standard. Immature spermatozoa from the caput epididymis (with minimal motility) were then co-incubated for 3 h at 38°C with the epididymosome samples (sperm cell concentration=8x106 sperm/mL). Controls included sperm cells from caput and cauda epididymides incubated in base medium. Percentage of motile spermatozoa and forward progressive motility (scale 0=non-motile, 5=fast and straight motility) were assessed using standard methods developed in our laboratory (Howard et al., 1990; Terrell et al., 2011) every 30 min and then normalized to 100% motility or FPM at time 0 for each sample. Acrosome integrity also was assessed before and after the 3-h co-incubation via fixation in paraformaldehyde and stained with Coomassie Brilliant Blue R-250 (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). Proportion of sperm cells with intact

acrosomes were recorded for each treatment, analyzing two hundred cells total per treatment of each individual male (Long et al., 1996).

Experimental design and statistical analysis

For initial immunostaining of tissues and isolated spermatozoa, 4 males were used on 4 different days (4 replicates). For Western Blot and mass spectrometry, 4 tissue samples from 2 males each, 4 epididymal sperm samples from 10 males each, and 4 samples of luminal fluid and epididymosomes from 5 males each were used in 4 replicates. For electron microscopy, 1 pooled sample of 5 males each were used. A total of 10 photographs were captured to ensure accurate assessment. For co-incubations, spermatozoa were collected from 6 different males and incubated with luminal fluid and epididymosomes isolated from 6 pools of 5 males. Motility and acrosome integrity was assessed in 4 different males and the spermatozoa of each male incubated with epididymosomes isolated from 4 pools of 5 males.

Statistical analyses were conducted using SAS software version 9.3 and GraphPad Prism software version 6. The proportion of positively stained spermatozoa following each treatment and the corresponding intensity of immunofluorescent stain was analyzed by analysis of variance with results further compared by protected Tukey's test, blocking for individual variation. Changes in percent motile spermatozoa, forward progressive movement, and caput sperm acrosome integrity were analyzed using analysis of variance with repeated measures, followed by protected Tukey's test, with blocking for

individual variation. Change in cauda sperm acrosome integrity was analyzed using a paired t-test.

Results

Presence and localization of cenexin in testicular tissues, epididymal segments, luminal fluid, and sperm cells.

Cenexin was detected in all seminiferous tubules and interstitial cells of testicular tissues, in epithelial cells from caput, corpus, and cauda epididymides and in the vas deferens (Figure 3.1). When epididymal sperm cells were stained for cenexin, the labelling was located in the centrosomal area of the sperm neck/mid-piece (Figure 3.1). Western Blot analysis (as well as mass spectrometry; data not shown) confirmed the presence of cenexin in tissues and in sperm cells isolated from the epididymis (Figure 3.2). Luminal fluids recovered from the different segments of the epididymis also contained cenexin (Figure 3.2). Epididymosomes isolated from luminal fluids in all segments of the epididymis contained cenexin (Figure 3.2). Electron microscopy was conducted to confirm that epididymosome fractions were free of contamination (Figure 3.3).

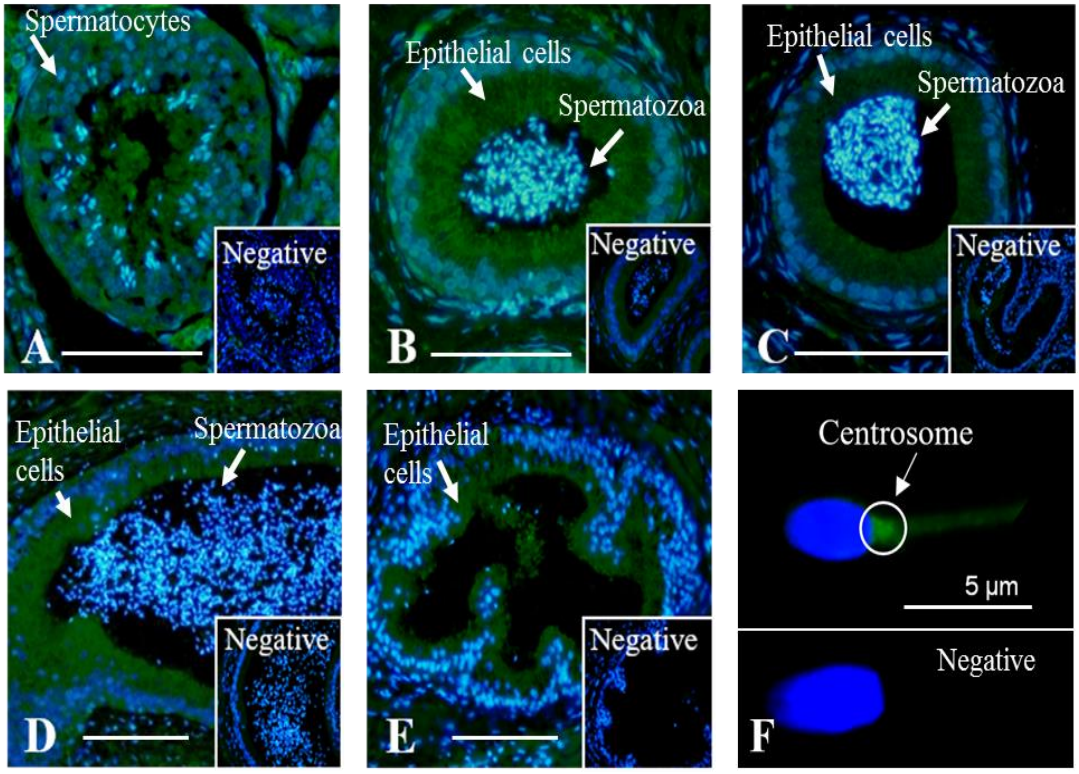

Figure 3.1. Localization of cenexin by immunofluorescence (FITC; insets show negative controls) and nuclear chromatin counterstaining (Hoechst) in cross sections of A) Seminiferous tubules, B) Caput epididymis, C) Corpus epididymis, D) Cauda epididymis, E) Vas Deferens (scale bars = 100µm) and F) in sperm centrosome from a cell collected in the cauda epididymis.

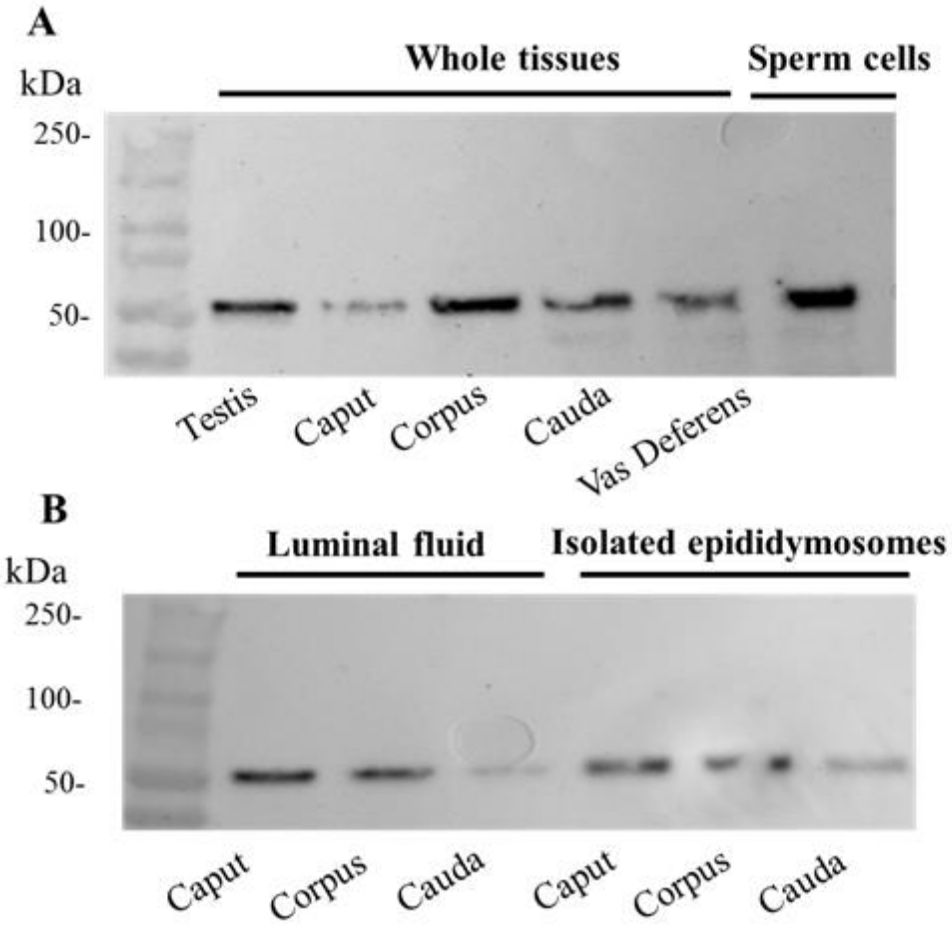

Figure 3.2. Cenexin detection by Western Blots in A) whole testicular and epididymal tissues as well as pooled sperm samples from epididymal segments and B) luminal fluid and isolated epididymosomes from epididymal segments.

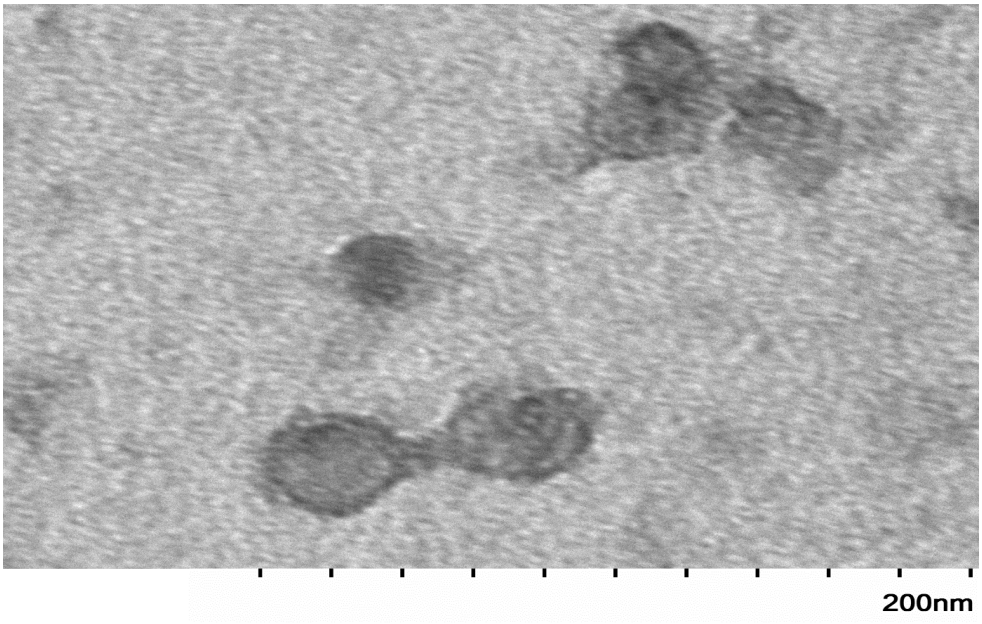

Figure 3.3. Transmission electron micrograph of isolated epididymosome fraction.

Role of epididymosomes in the transfer of cenexin between epididymal epithelium and sperm cells.

Percentages of spermatozoa containing cenexin were higher ($P < 0.05$) in cells isolated from the cauda epididymis (86.2 ± 7.0%) compared to testicular sperm cells (7.6 ± 1.6%; Figure 3.4). The average proportion of sperm cells with cenexin detected at the centrosome significantly increased ($P < 0.05$) in testicular spermatozoa co-incubated with epididymosome fractions (60.5 ± 4.3%) compared to controls (7.6 ± 1.6%) and in sperm samples co-incubated with base medium (12.6 ± 1.9%) or with luminal fluid (34.0 ± 8.6%; Figure 3.4). Fluorescence intensity in positive cells did not differ between the treatment groups ($P > 0.05$); however, the intensity in immature sperm cells co-incubated with epididymosome fractions tended to be similar to the positive controls (Figure 3.5).

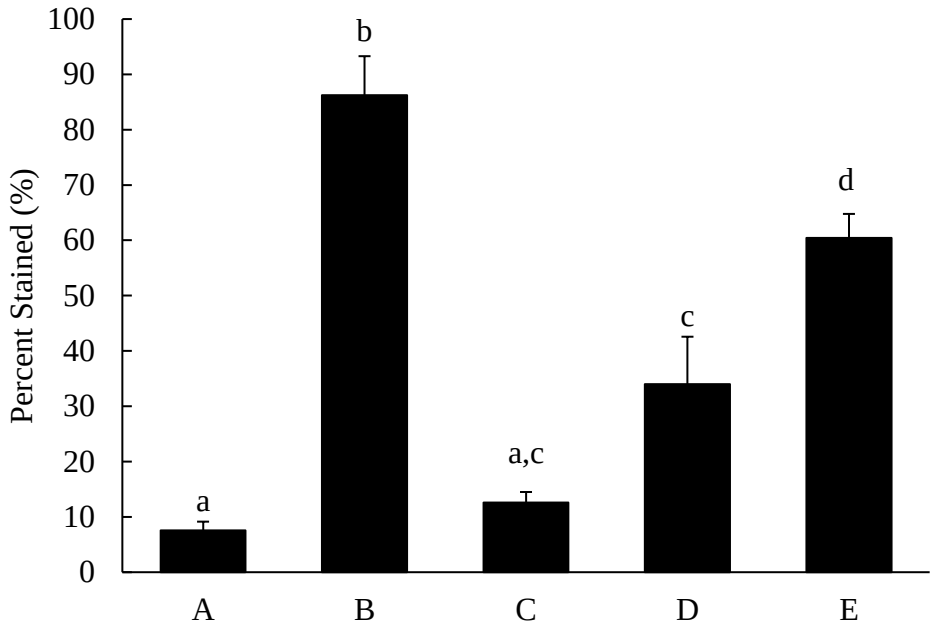

Figure 3.4. Percentages (mean ± SEM) of spermatozoa, showing staining for cenexin. Testicular spermatozoa non-exposed to epididymosomes (control, A) and mature spermatozoa (positive control, B). Testicular spermatozoa incubated for 3 h with: base medium (C), luminal fluid (D), or epididymosome fraction (E). Bars with different letters significantly differ ($P<0.01$).

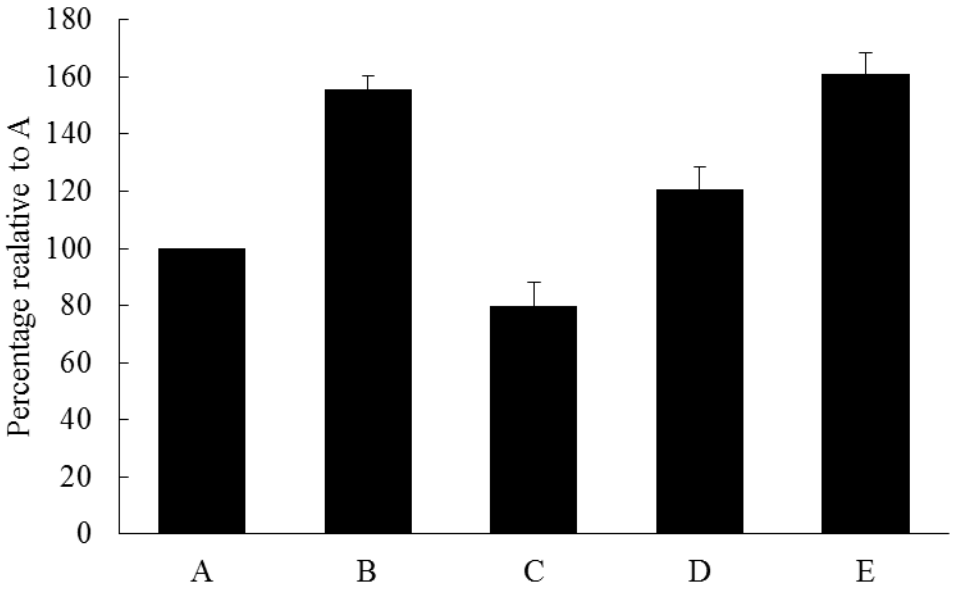

Figure 3.5. Relative fluorescence intensity (mean ± SEM) of sperm cells positively stained for cenexin compared to the untreated control testis sample (A). Testicular spermatozoa non-exposed to epididymosomes (control, A) and mature spermatozoa (positive control, B). Testicular spermatozoa incubated for 3 hrs with: base medium (C), luminal fluid (D), or epididymosome fraction (E).

Influence of epididymosomes on the sperm motility and acrosome integrity.

Exposure of immature sperm cells to epididymosomes resulted in sustained motility throughout incubation compared to immature sperm cells incubated in base medium or the control ($P < 0.05$, Figure 3.6). Forward progressive motility was altered by the epididymosome exposures at 1.5, 2.5, and 3 h of incubation ($P < 0.05$; Figure 3.6). There were no significant effects of incubations on percentages of sperm cells displaying an intact acrosome regardless of the treatment ($P > 0.05$, range 80-93%).

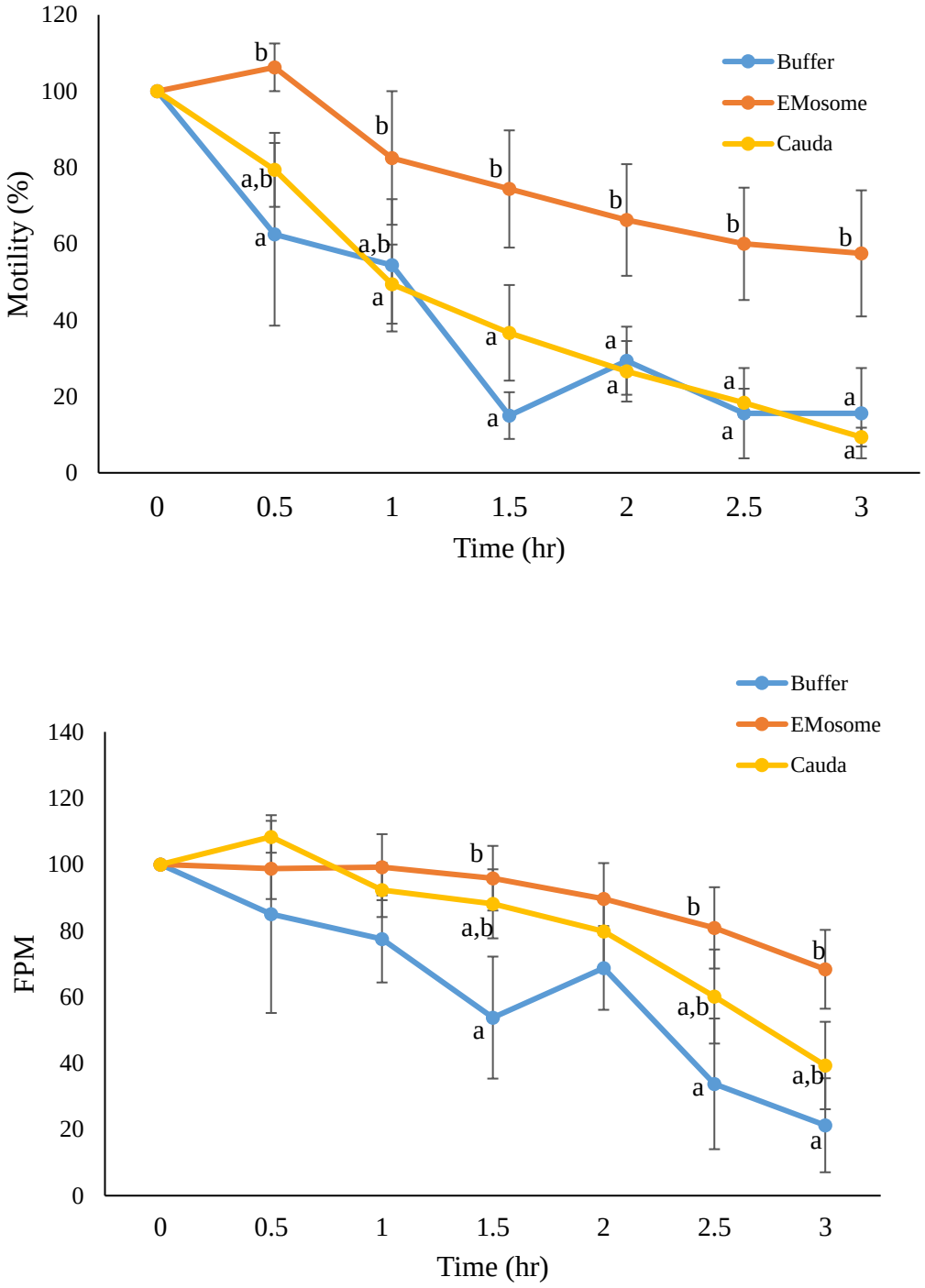

Figure 3.6. Percentages (mean ± SEM) of sperm motility (A) and forward progressive motility (FPM; B) normalized to 100 at time 0 in samples of matured spermatozoa

(Cauda; positive control), immature spermatozoa incubated with base medium (Buffer; negative control), or incubated with epididymosomes (Emosomes). Within time points, different letters indicate significant difference ($P<0.05$).

Discussion

The key finding from this study is the evidence for the transfer of cenexin – a maturational marker - from the epididymal epithelium to sperm cells via epididymosomes. Our results also provide evidence that epididymosomes may have multiple roles, such as increasing sperm cell protein content and sustaining sperm motility without influence on acrosome integrity. Analysis of tissues and sperm cells showed that cenexin is expressed in the epithelial cells of the epididymis, consistent with observations made in other somatic cells (Ishikawa et al., 2005). Detection of cenexin in sperm cells confirmed earlier studies from our laboratory showing that proportions of sperm cells with cenexin increase throughout epididymal transit (Rowlison et al., 2017) and could serve as a marker and possibly an index of centrosomal maturation (Ishikawa et al., 2005). Principal cells of the epididymal epithelium have previously been reported to secrete numerous proteins via epididymosomes involved in the sperm maturation process in the rat (Belleannée et al., 2009; Zhen et al., 2009; Suryawanshi et al., 2012). Likewise, cenexin was detected in the luminal fluid and more specifically in epididymosomes isolated from the epididymal segments, suggesting these cells are a principal source of cenexin from the epithelium. While cenexin has been previously reported to weigh approximately 95 kDa in the mouse (Hüber and Hoyer-Fender, 2007), our analyses in the cat (confirmed by gel digest and LC-MS/MS) revealed a smaller protein weighing 50 kDa. This difference in molecular weight may be due to post-translational modification of the protein

specific to the domestic cat, or possible proteolytic cleavage during sample processing.

Further, our studies strongly suggest that cenexin is transported via epididymosomes to the sperm cells, based on the significant increase in centrosome localized cenexin in the samples co-incubated with small vesicles. This is also supported by the fact that cenexin does not contain its own signal peptide sequence (as determined by sequence analysis via SignalP, version 4.0, D-score= 0.117; Petersen et al., 2011). Indeed, proteins that do not contain a signal sequence are not secreted from epididymal epithelial cells via the merocrine pathway, and instead are packaged and transported to the sperm cells within the epididymosome vesicles (Hermo and Jacks, 2002). Similarly, other proteins including aldolase reductase, p26h, macrophage migratory inhibition factor, and methylmalonate-semialdehyde dehydrogenase that lack a signal peptide were documented to be delivered via epididymosomes (Sullivan et al., 2007; Légaré et al., 1999; Suryawanshi et al., 2012). Together, these results indicate that the epididymosomes supplied cenexin, and potentially other proteins important to centrosomal maturation. Further proteomic analyses currently are underway in our laboratory to determine the exact composition of these epididymosomes. The exact role of each epididymal segment in production and secretory pattern still needs to be investigated in the cat model.

Acquiring motility is one of the key properties gained by sperm cells as they transit through the epididymis. Influence of epididymosomes on this

functional acquisition is not well understood; however, previous research indicated a supportive role (Sullivan et al., 2007; Girouard et al., 2011). Exposure of immature sperm cells to epididymosomes resulted in sustained motility throughout the 3-h incubation, thus indicating that the epididymosomes may be supplying proteins that promote motility. The same beneficial effect was observed on the forward progressive motility which suggest that epididymosomes also could supply nutrients or serve a mechanistic role in facilitating metabolism (Saez et al., 2003; Caballero et al., 2013). While previous studies have reported that a subpopulation of epididymosomes may provide proteins enhancing the sperm-egg interaction (Caballero et al., 2013; Girouard et al., 2011; Saez et al., 2003) we did not observe visible effects of co-incubation on the acrosome integrity. More analysis will be conducted in our laboratory to assess the effects of co-incubation on sperm-egg binding *in vitro*. Our experiments involved epididymosome suspensions that were more concentrated than in the physiological luminal fluid. As such, we hypothesize that the high concentration only accelerated the incorporation (in 3 h *in vitro* vs. several hours or days *in vivo* (Axnér, 2006) thereby providing insight into the physiological process, albeit at a faster rate.

In summary, our study demonstrates for the first time the secretion of cenexin throughout the epididymis and its incorporation by immature spermatozoa. Understanding of these physiological processes provides opportunities to enhance assisted reproductive techniques for effective *in vitro* sperm maturation. Impaired centrosomal maturation is a source of infertility in

the cat, human and cattle (Comizzoli et al., 2006; Rawe et al., 2002; Navara et al., 1996), but the ability to rescue normal functions of this organelle has yet to be achieved. Findings of this study provide important data pertinent to *in vitro* culture strategies to overcome infertility.

Conclusion

This study characterized the process in which cenexin is secreted by the male reproductive tract and incorporated by the sperm cell throughout epididymal transit. This is a first clear demonstration that a centrosomal protein is incorporated by the sperm cell as it undergoes maturation within the epididymis. This study also demonstrated a functional effect of epididymosome exposure on the acquisition of motility, indicating that the vesicles are also supplying crucial factors which are necessary for sperm cell movement. Together, the development of the centrosome and acquisition of motility will aid in the sperm cell's ability to achieve successful fertilization. These results improve our understanding of the underlying mechanisms of the maturation process and may contribute to the future development of assisted reproductive techniques for many species, including endangered felids.

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References

Axnér, E. (2006). Sperm maturation in the domestic cat. Theriogenology, 66(1), 14-24.

Belleannée, C., Silva, N. D., Shum, W. W. C., Marsolais, M., Laprade, R., Brown, D., & Breton, S. (2009). Segmental expression of the bradykinin type 2 receptor in rat efferent ducts and epididymis and its role in the regulation of aquaporin 9. Biology of reproduction, 80(1), 134-143.

Caballero, J. N., Frenette, G., Belleannée, C., & Sullivan, R. (2013). CD9-positive microvesicles mediate the transfer of molecules to bovine spermatozoa during epididymal maturation. PloS one, 8(6), e65364.

Comizzoli, P., Wildt, D. E., & Pukazhenthi, B. S. (2006). Poor centrosomal function of cat testicular spermatozoa impairs embryo development in vitro after intracytoplasmic sperm injection. Biology of reproduction, 75(2), 252-260.

Cooper, T. G. (1998). Interactions between epididymal secretions and spermatozoa. Journal of reproduction and fertility. Supplement, 53, 119-136.

Dacheux, J. L., & Dacheux, F. (2014). New insights into epididymal function in relation to sperm maturation. Reproduction, 147(2), R27-R42.

Dacheux, Jean-Louis, Clémence Belleannée, Benoit Guyonnet, Valérie Labas, Ana-Paula Teixeira-Gomes, Heath Ecroyd, Xavier Druart, Jean-Luc Gatti, and Françoise Dacheux. (2012). The contribution of proteomics to understanding epididymal maturation of mammalian spermatozoa. Systems biology in reproductive medicine, 58(4), 197-210.

Fornes, M. W., Barbieri, A., & Cavicchia, J. C. (1995). Morphological and enzymatic study of membrane-bound vesicles from the lumen of the rat epididymis. Andrologia, 27(1), 1-5.

Frenette, G., & Sullivan, R. (2001). Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. Molecular reproduction and development, 59(1), 115-121.

Girouard, J., Frenette, G., & Sullivan, R. (2011). Comparative proteome and lipid profiles of bovine epididymosomes collected in the intraluminal compartment of the caput and cauda epididymidis. International journal of andrology, 34(5pt2).

Hermo, L., & Jacks, D. (2002). Nature's ingenuity: bypassing the classical secretory route via apocrine secretion. Molecular reproduction and development, 63(3), 394-410.

Howard, J.G., Brown, J.L., Bush, M., & Wildt, D.E. (1990). Teratospermic and Normospermic Domestic Cats: Ejaculate Traits, Pituitary—Gonadal Hormones, and Improvement of Spermatozoal Motility and Morphology After Swim-Up Processing. Journal of andrology, 11(3), 204-215.

Hüber, D., & Hoyer-Fender, S. (2007). Alternative splicing of exon 3b gives rise to ODF2 and Cenexin. Cytogenetic and genome research, 119(1-2), 68-73.

Ishikawa, H., Kubo, A., Tsukita, S., & Tsukita, S. (2005). Odf2-deficient mother centrioles lack distal/subdistal appendages and the ability to generate primary cilia. Nature cell biology, 7(5), 517-524.

Kirchhoff, C., & Hale, G. (1996). Cell-to-cell transfer of glycosylphosphatidylinositol-anchored membrane proteins during sperm maturation. MHR: Basic science of reproductive medicine, 2(3), 177-184.

Légaré, C., Bérubé, B., Boué, F., Lefièvre, L., Morales, C. R., El-Alfy, M., & Sullivan, R. (1999). Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein. Molecular reproduction and development, 52(2), 225-233.

Long, J. A., Wildt, D. E., Wolfe, B. A., Critser, J. K., DeRossi, R. V., & Howard, J. (1996). Sperm capacitation and the acrosome reaction are compromised in teratospermic domestic cats. Biology of reproduction, 54(3), 638-646.

Morales, A., & Cavicchia, J. C. (1991). Release of cytoplasmic apical protrusions from principal cells of the cat epididymis, an electron microscopic study. Tissue and cell, 23(4), 505-513.

Navara, C. S., First, N. L., & Schatten, G. (1996). Phenotypic variations among paternal centrosomes expressed within the zygote as disparate microtubule lengths and sperm aster organization: correlations between centrosome activity and developmental success. Proceedings of the National Academy of Sciences, 93(11), 5384-5388.

Nicander, L., & Malmqvist, M. (1977). Ultrastructural observations suggesting merocrine secretion in the initial segment of the mammalian epididymis. Cell and tissue research, 184(4), 487-490.

Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature methods, 8(10), 785-786.

Rawe, V. Y., Terada, Y., Nakamura, S., Chillik, C. F., Olmedo, S. B., & Chemes, H. E. (2002). A pathology of the sperm centriole responsible for defective sperm aster formation, syngamy and cleavage. Human Reproduction, 17(9), 2344-2349.

Rowlison, T., Ottinger, M. A., & Comizzoli, P. (2017). Deciphering the mechanisms involving cenexin, ninein and centriolin in sperm maturation during epididymal transit in the domestic cat. Reproduction in Domestic animals, 52(S2), 193-196.

Saez, F., Frenette, G., & Sullivan, R. (2003). Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. Journal of andrology, 24(2), 149-154.

Sullivan, R., Frenette, G., & Girouard, J. (2007). Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. Asian journal of andrology, 9(4), 483-491.

Sullivan, R., & Saez, F. (2013). Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. Reproduction, 146(1), R21-R35.

Suryawanshi, A. R., Khan, S. A., Joshi, C. S., & Khole, V. V. (2012). Epididymosome-Mediated Acquisition of MMSDH, an Androgen-Dependent and Developmentally Regulated Epididymal Sperm Protein. Journal of andrology, 33(5), 963-974.

Terrell, K.A., Wildt, D.E., Anthony, N.M., Bavister, B.D., Leibo, S.P., Penfold, L.M., Marker, L.L. and Crosier, A.E. (2011). Glycolytic enzyme activity is essential for domestic cat (Felis catus) and cheetah (Acinonyx jubatus) sperm motility and viability in a sugar-free medium. Biology of reproduction, 84(6), 1198-1206.

Thimon, V., Koukoui, O., Calvo, E., & Sullivan, R. (2007). Region-specific gene expression profiling along the human epididymis. Molecular human reproduction, 13(10), 691-704.

Zhen, W., Li, P., He, B., Guo, J., & Zhang, Y. L. (2009). The novel epididymis-specific beta-galactosidase-like gene Glb1l4 is essential in epididymal development and sperm maturation in rats. Biology of reproduction, 80(4), 696-706.

Chapter 4: Sperm centrosome function is acquired via epididymal factors and may be a source of infertility in the domestic cat.

Abstract

Maturation of the spermatozoa during epididymal transit is associated with the integration of specific proteins and acquisition of functionality. Using the domestic cat model (*Felis catus*), we previously demonstrated that secreted epididymal vesicles termed, epididymosomes, supply key proteins to the developing spermatozoa and exposure to these vesicles enhance motility. Additionally, we have demonstrated that these vesicles aid in the maturation of the sperm centrosome, an organelle which functions to complete pronuclear fusion after oocyte penetration. The objectives of this study were to (1) assess the role of epididymosomes on the acquisition of fertilization capabilities of maturing sperm cells (2) assess the presence of sperm centrosome abnormalities in the domestic cat model and (3) determine the impact of these abnormalities on the success of fertilization. Co-incubation of immature sperm cells for 1 h and 15 min with epididymosomes demonstrated an increasing trend in pronuclear fusion. This study also assessed the impact of malformed spermatozoa, specifically at the centrosome, on fertilization success. Results showed diminished rates of pronuclear fusion in samples with a high proportion of abnormalities. These findings provide evidence that improper development and maturation of the sperm centrosome may be an

important source of infertility in the domestic cat model. Establishing this method of diagnosis in the domestic cat will aid in the ability to determine this source of infertility in other species including humans and endangered wild cats. In summary, these results will enhance future efforts to create assisted reproductive techniques to overcome this type of infertility with particular potential for wild felid conservation.

Introduction

Proper maturation of the spermatozoa throughout epididymal transit is essential to acquiring the ability to successfully penetrate an oocyte and undergo the first stages of cellular division (Dacheux and Dacheux, 2014). Understanding the physiological changes that the sperm cell undergoes is critical for the development of assisted reproductive techniques that may overcome problems with infertility or the development of efficient contraceptive strategies. Beside important morphological changes, sperm maturation is associated with the integration of specific factors including key peptides and microRNA (Dacheux and Dacheux, 2014; Sullivan R & Saez, 2013).

The classical method of protein secretion is the merocrine pathway in which proteins contain signal sequences and are secreted individually from the epithelium. Once secreted into the luminal fluid the proteins then bind with the sperm surface and be integrated by the cell. Another method of secretion has recently been identified in which proteins without a signal sequence are secreted

within membranous vesicles termed, “epididymosomes” (Hermo and Jacks, 2002). This type of secretory process has been identified in a number of species including the rat (*Rattus rattus*; Fornes et al., 1995), hamster (*Mesocricetus auratus*; Legare et al., 1999), bovine (*Bos taurus*; Frenette and Sullivan, 2001), human (*Homo sapiens*; Mesocricetus auratus; Thimon et al., 2007) and cat (Morales and Cavicchia, 1991). While epididymosomes have been observed in domestic cat, little is known regarding their role on sperm cell maturation (Morales and Cavicchia, 1991). Previously studies demonstrated that exposing immature sperm cells to epididymosomes improved motility (Chapter 3); however, investigations into the impact of epididymosome exposure on the ability of the sperm cell to successfully fertilize an oocyte has not yet been investigated in any species to the best of our knowledge.

A critical aspect of sperm cell maturation is the acquisition of a functional centrosome. The sperm centrosome is an essential organelle playing a key role just after penetration into the oocyte. It serves to organize the sperm aster which is required for proper syngamy and formation of the first mitotic spindle. The centrosome is located at the base of the sperm head and, following oocyte penetration, will migrate to the center of the embryo to produce a mass array of microtubules, collectively referred to as the sperm aster. These microtubules will bind with the male and female pronuclei and physically draw them together for pronuclear fusion (Schatten, 1994; Navara et al., 1996). Our laboratory previously demonstrated the importance of centrosome maturation by injecting domestic cat oocytes with either mature, ejaculated spermatozoa or

immature spermatozoa collected from the testis segment (Comizzoli et al., 2006). Oocytes injected with mature spermatozoa demonstrated formation of a large, competent sperm aster. Conversely, injection of immature spermatozoa resulted in small asters and significantly increased time for the onset of first cleavage and embryonic death. These differences point to key maturational processes that occur specifically at the centrosome while the whole sperm cell is undergoing maturation within the epididymis. Improper maturation has been associated with infertility in species such as the human and bovine (Rawe et al., 2002; Navara et al., 1996) in which the injection of spermatozoa collected from infertile individuals resulted in diminished aster production and pronuclear fusion

Research in human fertility has demonstrated that the function of the centrosome may be gauged through the assessment of sperm morphology. Chemes and coworkers (1999) observed that a significant proportion of sperm cells collected from infertile men displayed abnormalities in the head tail junction where the centrosome is located. The flagellum in these sperm types appeared to be misaligned and displayed increased fragility wherein the flagellum would readily dissociate from the rest of the cell. Rawe et al. (2002) demonstrated that specifically selecting sperm with properly aligned head-tail junctions from an infertile male collection increased the likelihood of proper

aster formation, pronuclear fusion, and cleavage, indicating improved function of the centrosome.

Of 36 wild felid species, 18 are classified as endangered according to the International Union for the Conservation of Nature (IUCN Red List, 2017). Many of these species exhibit teratospermia, a condition in which the majority of sperm cells display abnormalities (≥ 60% morphological abnormalities) that impede the ability to achieve fertilization (Pukazhenthi et al., 2006). While many of the abnormalities have been extensively studied, complications with the sperm centrosome have not been investigated in any felid species. It should be noted that morphological abnormalities of the whole midpiece region that encompass the head-tail junction have been commonly observed in many felids (Pukazhenthi et al., 2006).

The objectives of the study were to (1) assess the role of epididymosomes on the acquisition of fertilization capabilities of developing sperm cells (2) assess the presence of sperm centrosome abnormalities in the domestic cat model and (3) determine the impact that these abnormalities may have on the success of fertilization.

Materials and Methods

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Sample collection and preparation

Adult (>1 year) domestic cat testis and ovary samples were supplied by local veterinary clinics following routine ovario-hysterectomy and orchiectomy. Tracts were transported and stored in phosphate buffered saline (PBS) at 4°C until processing. Epididymal tissues were dissected in Hepes-Ham medium F10 (25 mM Hepes, 1.0 mM pyruvate, 2.0 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 5% fetal calf serum) using a scalpel blade and kept separately. The epididymal tissue sample was sliced with a scalpel blade to recover the luminal fluids containing epididymosomes and placed into plain Hepes-Ham medium F-10, without fetal bovine serum. Cell debris was discarded from the supernatant by a series of centrifugations at 700 x g for 10 min and 3,000 x g for 10 min at room temperature. The epididymosome fraction was isolated from the remaining luminal fluid by ultracentrifugation at 100,000 x g for 2 h at 4°C and re-suspended in fresh Ham's F-10 media. Aliquots of epididymosome samples were stored at -20°C until further processing.

Grade I immature oocytes were identified as having homogeneous dark cytoplasm and several layers of compacted cumulus, and were isolated after slicing ovaries in dissecting medium (Eagle MEM with Hank's balanced salt-

Gibco Laboratories, supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES and 0.1% bovine serum albumin). Oocytes were then cultured for 28 h in *in vitro* maturation (IVM) medium composed of Sage blastocyst medium (Pasadena, CA, USA) supplemented with 0.01% (v/v) FSH and 0.01% LH (National Hormone and Pituitary Program, Rockville, MD, USA) prior to *in vitro* fertilization.

Electron microscopy

Pellets obtained after ultracentrifugation were stained with uranyl acetate for 1 min and observations performed using a transmission electron microscope (Zeiss 10 CA Transmission Electron Microscope) at the University of Maryland Laboratory for Biological Ultrastructure, USA, to confirm presence of epididymosomes without tissue contamination following a previously established protocol (Chapter 3).

Epididymosome co-incubation and centrosome assessment

Epididymosome samples were diluted to a concentration of 4μg total protein/ μL in Ham's F-10 medium as determined by BioRad assay kit using bovine serum albumin as standard. Immature caput spermatozoa were then co-incubated for 1 h and 15 min at 38°C with epididymosome samples in Ham's F-10 medium supplemented with 5% fetal bovine serum. Controls included sperm cells from caput and cauda epididymides incubated in buffer medium, without epididymosomes. Spermatozoa were then centrifuged at 300 x g for 8 min and reconstituted with Sage blastocyst medium (cauda spermatozoa=5 x 10^6 motile sperm/mL and caput spermatozoa=8-12 x 10^6 sperm/mL) prior to *in*

vitro fertilization (20 oocytes minimum per treatment group). Oocytes and spermatozoa were co-incubated for 16.5 h then fixed in 4% paraformaldehyde overnight at 4°C.

After three washings in medium (phosphate buffered saline supplemented with 0.5% Triton-X, and 2% fetal bovine serum), non-specific binding sites were blocked in saturation medium (phosphate buffered saline supplemented with 20% fetal calf serum and 0.5% Triton-X) for 1hr at 38°C. Samples were then incubated with Hoechst staining solution (1 µg/mL) for 10 min, room temperature, before observation under a microscope fitted with epifluorescence (Olympus BX 41).

Proportion of oocytes successfully penetrated was assessed via presence of both male and female pronuclei. Success of sperm aster function was assessed by measuring the proximity of pronuclei: competent asters were identified as ones which were capable of physically drawing the pronuclei closer for fusion and were classified as either "close (pronuclei being ≤1µm in proximity) or fused", or "apart" (pronuclei being ≥1µm in proximity; Figure 4.1). Spot Basic 5.1 software (Diagnostics Instruments) was used to capture images of oocytes and record distance between pronuclei.

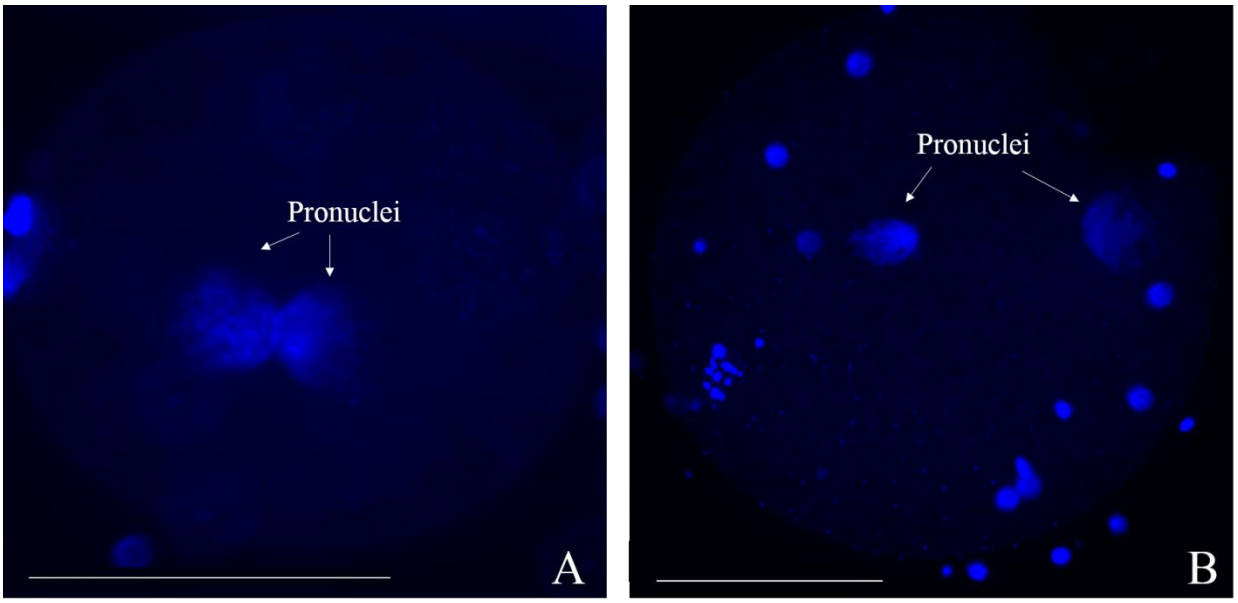

Figure 4.1. Examples of domestic cat oocytes with pronuclei classified as either A= "close (pronuclei being ≤ 1µm in proximity) or fused", or B= "apart" (pronuclei being ≥ 1µm in proximity). Scale bar= 100µm.

Centrosomal assessment of normo- versus teratospermic males

Adult cat testis and ovary samples were collected and processed following the procedures described above. Cauda epididymal tissue and sperm cells were dissected in Hepes-Ham medium F10 (25 mM Hepes, 1.0 mM pyruvate, 2.0 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 5% fetal bovine serum) with a scalpel blade and kept separately between individuals. Following collection, 10 µL of each sperm suspension was smeared on a glass slide and fixed with 4% paraformaldehyde (10 min, room temp) and stained with Coomassie Brilliant Blue R-250 (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). Normospermic males were categorized as having ≥60% spermatozoa of normal morphology as compared to teratospermic males which displayed ≥40% abnormal sperm morphology. Morphological defects included sperm cells that displayed coiled tails, double or missing heads, missing acrosomes, and abnormal midpieces. The proportion of spermatozoa which displayed abnormal morphology specifically at the location of the centrosome was also recorded for each individual.

Spermatozoa from the cauda epididymis were isolated from one normospermic and one teratospermic male per trial in Hepes-Ham medium F10 medium, centrifuged at 300 x g for 8 min and reconstituted with Sage blastocyst media (spermatozoa= 5 x 10^6 motile sperm/mL) prior to *in vitro* fertilization (20 oocytes minimum per treatment group). Oocytes and spermatozoa were co-

incubated for 16.5 h then fixed in 4% paraformaldehyde overnight at 4°C. Hoechst staining of oocytes and the success of penetration and aster function were also completed following the same procedures detailed above.

Experimental design and statistical analysis

For epididymosome co-incubations of immature sperm cells, spermatozoa were collected from n= 3 different males and incubated with epididymosomes isolated from 3 pools of 5 males. Twelve males total were assessed as either normospermic versus teratospermic. Assessment of fertilization and centrosome function was completed using one normospermic and one teratospermic male per trial, 3 trials total (n= 6 males total). Oocytes were isolated from 4-5 females per trial for both studies. Statistical analyses were conducted via SAS software version 9.3. The proportion of oocytes which were successfully penetrated, the subsequent proportion with "close or fused" pronuclei as well as the proportion of morphological abnormalities at the head-tail junction, was compared between treatment groups via analysis of variance with results further compared via protected Tukey's test, blocking for individual variation.

Results

Influence of epididymosomes on sperm fertilization.

Spermatozoa were isolated from the caput and cauda epididymis and the motility assessed (average caput spermatozoa= 16.7 ± 3.3% motility and 3.3 ±

0.3 forward progressive movement- FPM, cauda spermatozoa = 65.0 ± 7.6% motility and 3.7 ± 0.3 FPM; Figure 4.2). There was no significant difference in the proportion of successfully penetrated oocytes treatment groups in which caput sperm cells were exposed to epididymosomes (45.0 ± 16.1% penetrated oocytes) as compared to caput spermatozoa which were incubated in base medium alone (28.3 ± 9.0% penetrated oocytes; cauda spermatozoa= 32.7 ± 9.3% penetrated oocytes; P= 0.6011; Figure 4.3). The proportion of penetrated oocytes with pronuclei that were close in proximity (≤1 μm or less) or fused bordered on significance in the oocytes fertilized by the epididymosome-exposed caput spermatozoa (90.9 ± 9.1% oocytes) as compared to caput spermatozoa incubated in base medium (35.0 ± 17.7% oocytes; cauda spermatozoa= 88.9 ± 11.1% oocytes; P= 0.0562; Figure 4.4).

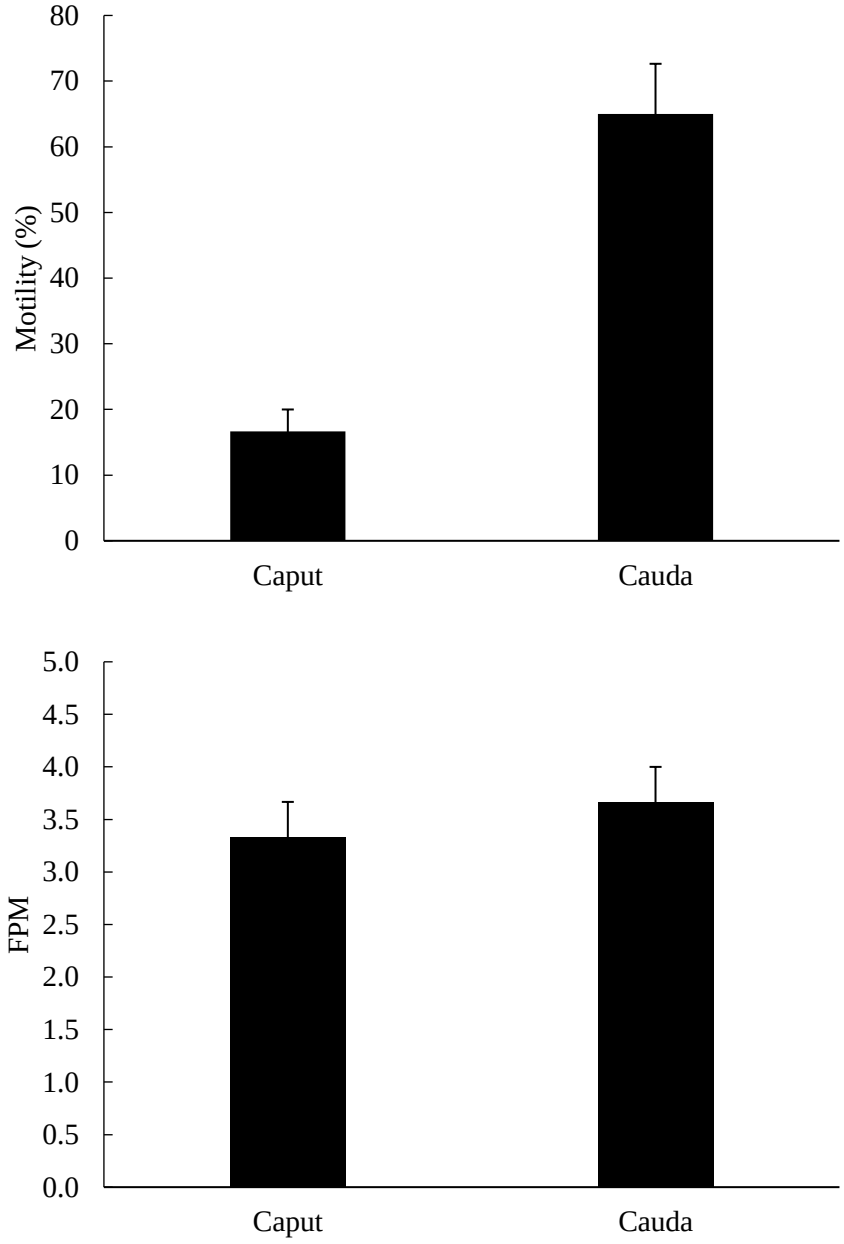

Figure 4.2. Motility and forward progressive movement (FPM; mean ± SEM) in samples of caput and cauda epididymal spermatozoa.

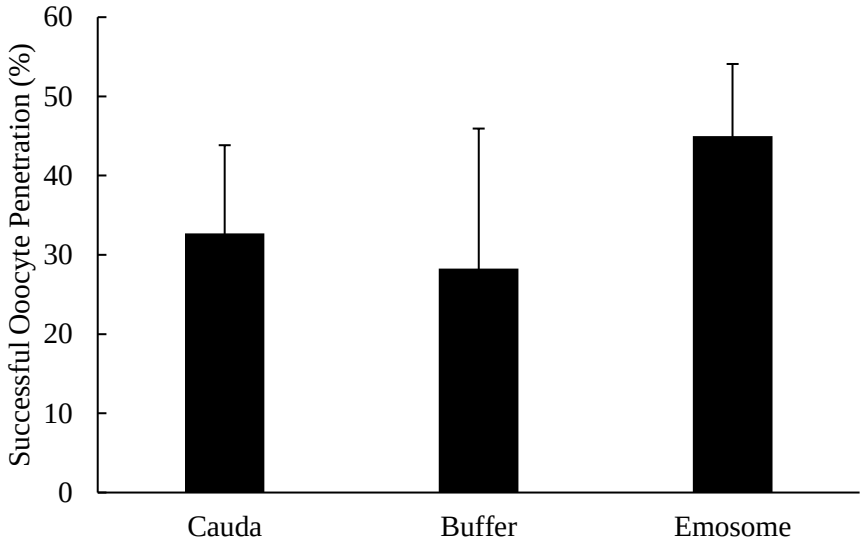

Figure 4.3. Proportion (mean ± SEM) of successfully penetrated oocytes following 16.5 h co-culture. Immature spermatozoa were either previously exposed to epididymosomes (Emosomes) or base medium (Buffer), as well as cauda epididymal spermatozoa (P= 0.6011).

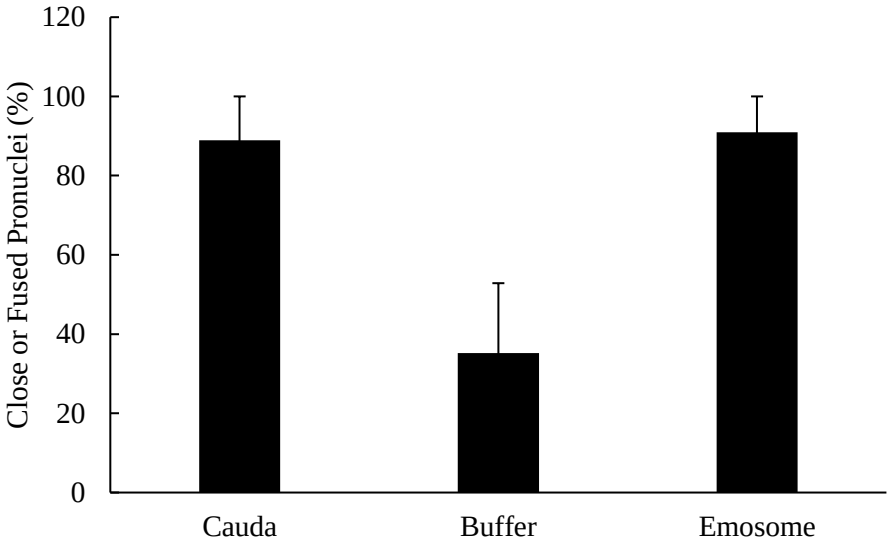

Figure 4.4. Proportion (mean ± SEM) of penetrated oocytes with pronuclei classified as "close (pronuclei being ≤ 1µm in proximity) or fused", following 16.5 h co-culture. Immature spermatozoa were either previously exposed to epididymosomes (Emosomes) or base medium (Buffer), as well as cauda epididymal spermatozoa (P= 0.0562).

Comparison of fertilization success between normospermic versus teratospermic males.

Spermatozoa were isolated from the cauda epididymis and the morphology at the location of the centrosome, the head-tail junction, assessed. Observed morphological abnormalities at the head-tail junction included sperm cells in which the head was dissociated or misaligned, abnormally thin or thickened head-tail junction, and fractured or discontinuous membrane at the head-tail junction (Figure 4.5). On average, the proportion of sperm cells with head-tail abnormalities was significantly higher in the teratospermic males (11.6 ± 2.5% abnormal spermatozoa) as compared to normospermic individuals (1. 4± 0.7% abnormal spermatozoa; P= 0.0074; Figure 4.6; a comprehensive list of abnormalities can be found in Appendix 1). A majority of the individuals analyzed had ≤10% abnormalities at the head-tail junction regardless of whether they were normospermic or teratospermic (83.3% of males analyzed, ten of the twelve males); however, the other two males were observed to have sperm samples in which 20 and 22% of the spermatozoa displayed abnormalities (16.7% of the males analyzed).

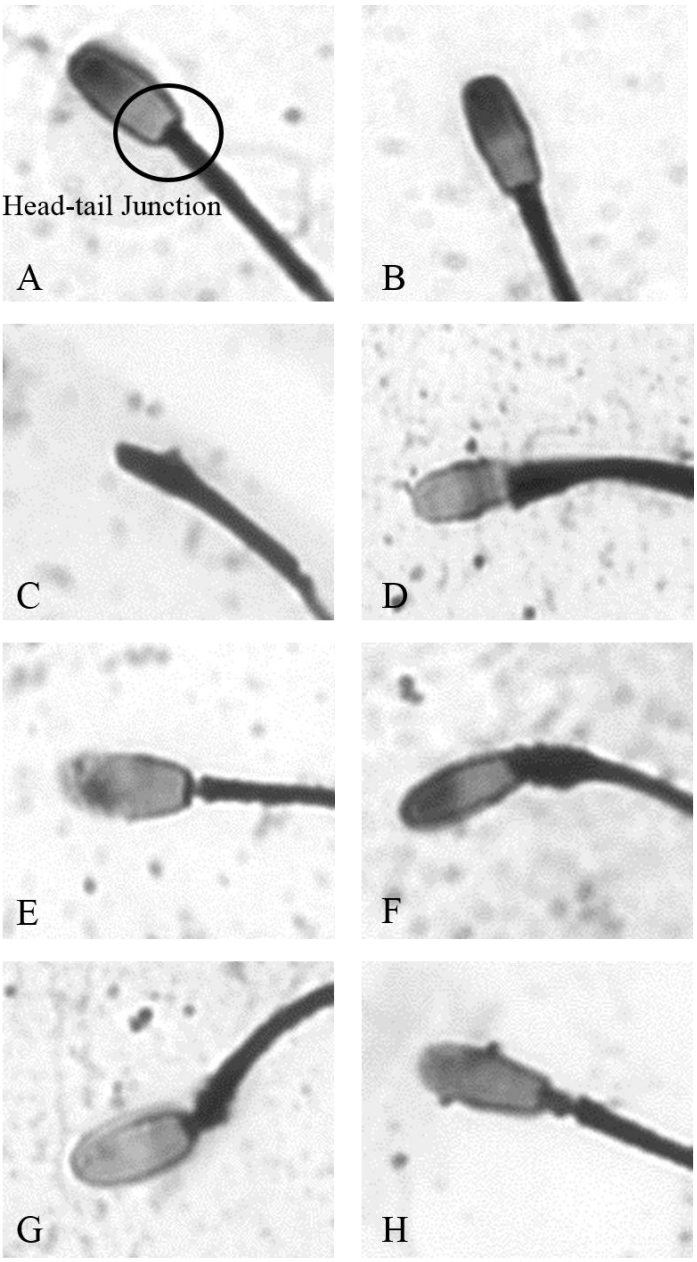

Figure 4.5. Examples of sperm cells with morphological abnormalities at the head-tail junction. A= diagram showing location of the centrosome, the head-tail junction, B= normal spermatozoa, C= missing head, D= misaligned head, E= thin head-tail junction, F= thick head-tail junction, G= fractured membrane, H= discontinuous membrane.

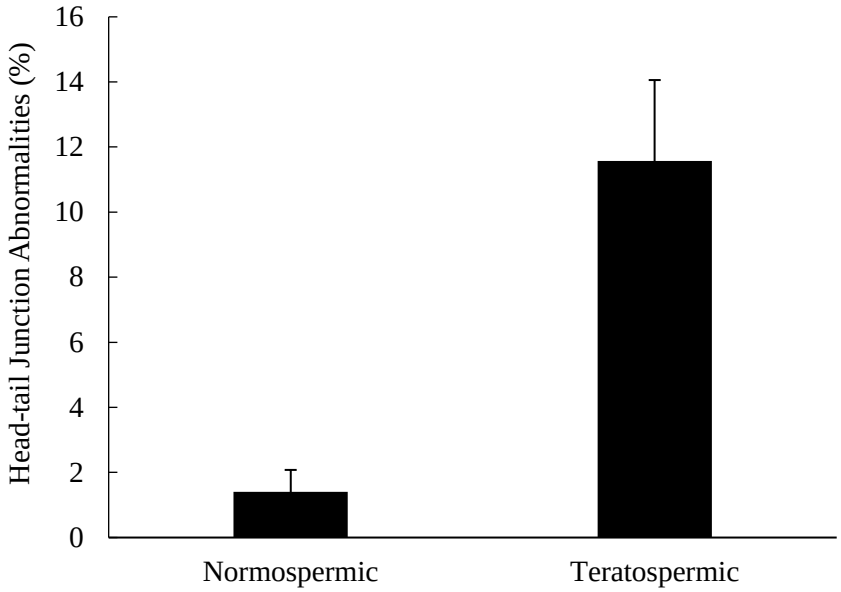

Figure 4.6. Proportion of total spermatozoa which displayed morphological abnormalities at the head-tail junction (mean ± SEM) in normospermic and teratospermic males ($P < 0.01$).

Spermatozoa were isolated from the cauda epididymis and the motility assessed (average normospermic male spermatozoa= 63.3 ± 12.0% motility and 3.2 ± 0.2 FPM, teratospermic male spermatozoa = 40.0 ± 10.0% motility and 2.8 ± 0.2 FPM; Figure 4.7). There was no significant difference in the proportion of successfully penetrated oocytes following IVF with spermatozoa isolated from normospermic males (39.2 ± 9.1% penetrated) as compared to teratospermic males (35.7 ± 15.7% penetrated; P= 0.5704; Figure 4.8). There was also no significant difference in the proportion of penetrated oocytes with pronuclei that were in close proximity (≤1 μm or less) or fused following IVF with normospermic males (100 ± 0% oocytes) as compared to teratospermic males (93.3 ± 6.7% oocytes; P= 0.3739; Figure 4.9).

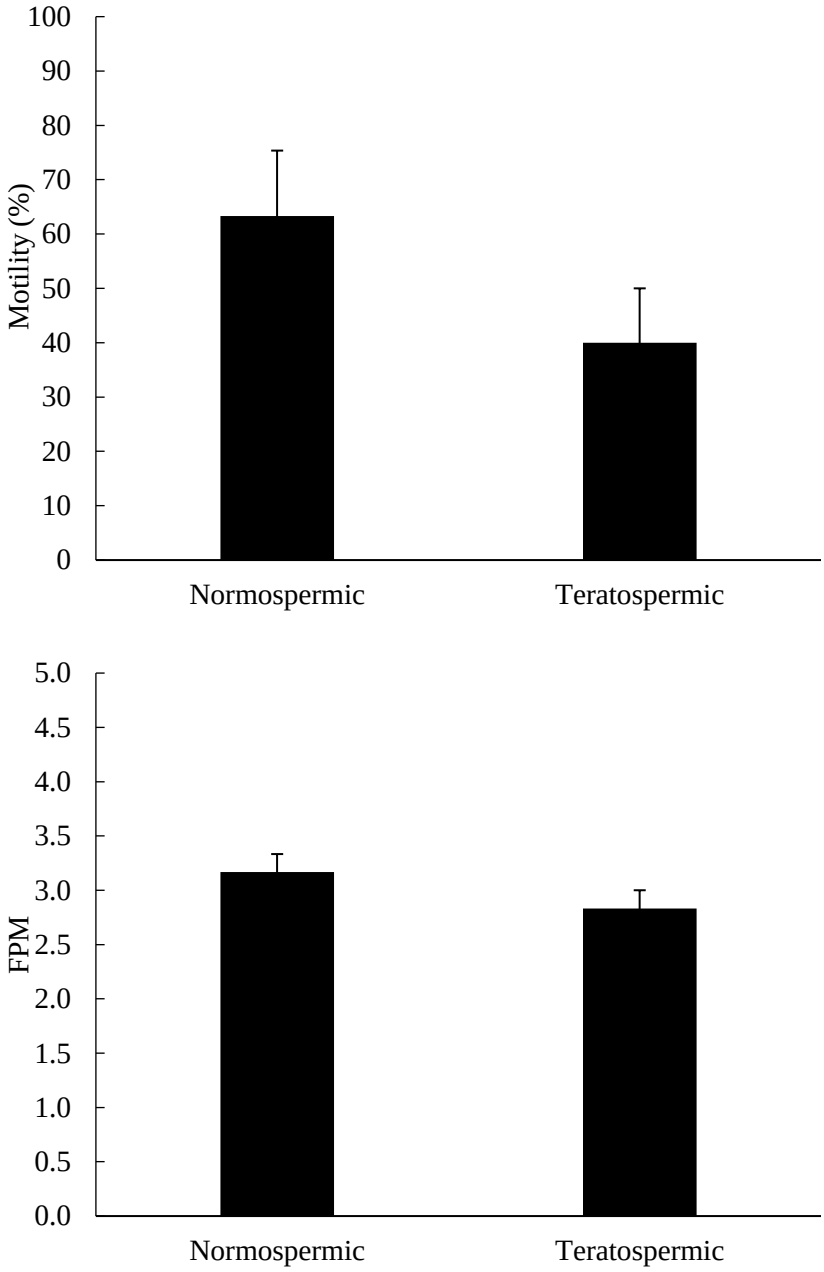

Figure 4.7. Motility and forward progressive movement (FPM; mean ± SEM) of cauda spermatozoa samples of normospermic and teratospermic males.

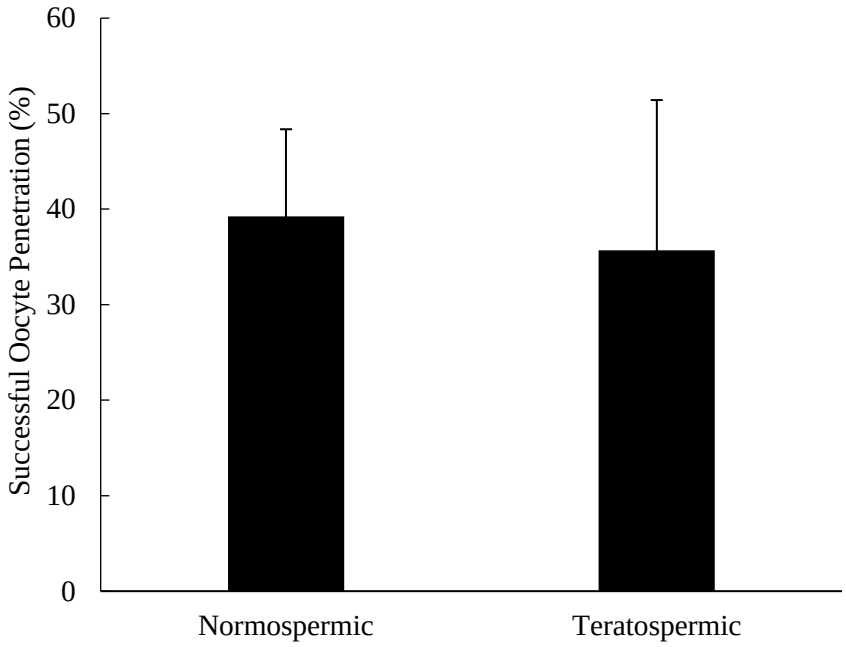

Figure 4.8. Proportion (mean ± SEM) of successfully penetrated oocytes following 16.5 h co-culture. Cauda spermatozoa were isolated from normospermic and teratospermic males (P= 0.5704).

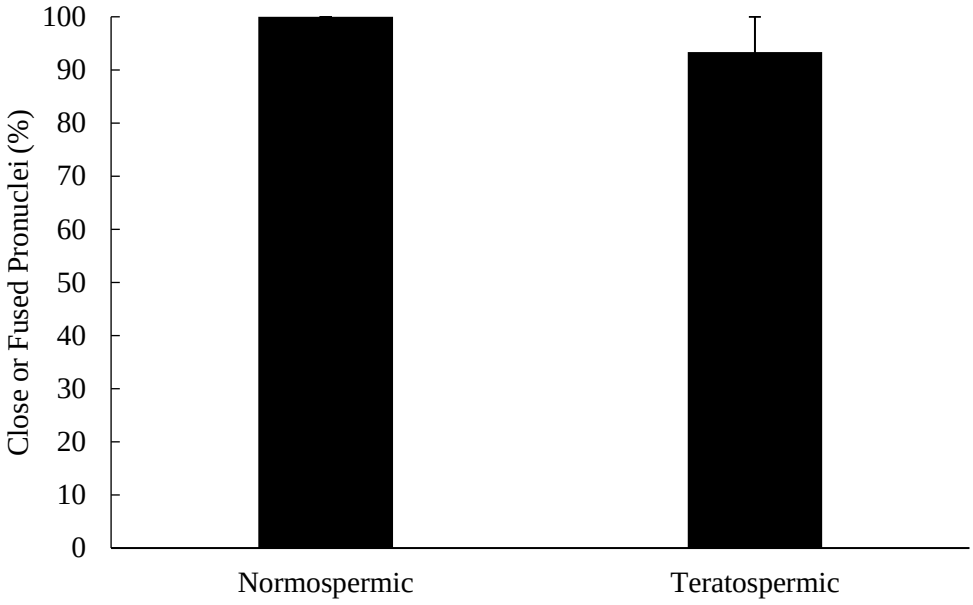

Figure 4.9. Proportion (mean ± SEM) of penetrated oocytes with pronuclei classified as "close (pronuclei being ≤ 1μm in proximity) or fused", following 16.5 h co-culture. Cauda spermatozoa were isolated from normospermic and teratospermic males (P= 0.3739).

Out of the six males, one individual had 22% spermatozoa with abnormalities at the head tail junction; the five other males had ≤7% abnormalities (average= 4.0 ± 1.1% abnormalities, range= 1-7% abnormalities). Following fertilization, using a sample from the male with 22% abnormal spermatozoa, there were 66.7% successfully penetrated oocytes; however, the oocytes with pronuclei that were in close proximity (≤1 μm or less) or fused decreased (80% of the penetrated oocytes, as compared to the other five individuals= 100 ± 0%; Figure 4.10).

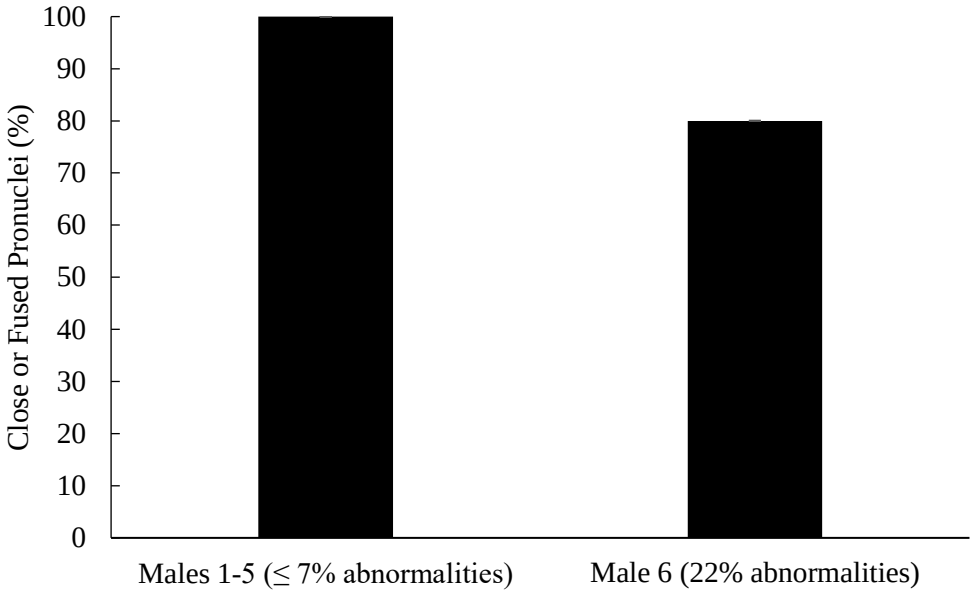

Figure 4.10. Proportion (mean ± SEM) of penetrated oocytes with pronuclei classified as "close (pronuclei being ≤ 1µm in proximity) or fused", following 16.5 h co-culture. Cauda spermatozoa were collected from males #1-5 which displayed ≤ 7% abnormalities at the head tail junction and male #6 which displayed 22% abnormalities.

Discussion

Acquiring the ability to bind and penetrate an oocyte as well as successfully undergo the first stages of cellular development is one of the key properties gained by sperm cells throughout the epididymal transit. Influence of epididymosomes on this functional acquisition is not well understood; however, previous research has indicated a supportive role (Caballero et al., 2013; Girouard et al., 2011; Saez et al., 2003). For example, the proteins, P34H, P26h, and P25b have been identified to associate with epididymosomes in the human, hamster, and bovine, respectively (Sullivan, 1999). These proteins are derived from orthologous genes between these species and function in zona pellucida recognition and binding during the acrosome reaction. Glutathione peroxidase 5 has also been identified as a protein associated with epididymosomes in the mouse, where it prevents premature acrosomal reaction (Rejraji et al., 2002). Exposure of immature sperm cells to epididymosomes in this study also showed a trend towards increased proportion of successfully penetrated oocytes.

The role of the centrosome is to produce a mass array of microtubules, collectively referred to as the sperm aster, which binds with the male and female pronuclei and physically draw them together for pronuclear fusion (Schatten, 1994; Navara et al., 1996). The competency of the centrosome to complete this task can be gauged by this size of the aster produced and timing to first cleavage as we have previously demonstrated in the domestic cat (Comizzoli et al., 2006). Mature sperm centrosomes produced a larger aster and achieved first

embryonic cleavage at a quicker rate as compared to immature spermatozoa. Similarly, the increased rate that the epididymosome-exposed sperm cells achieved pronuclear fusion in this study indicates that the centrosomes of these spermatozoa were more functionally competent as a result of the potential transfer of beneficial factors. Previously, we characterized the transfer of cenexin- a centrosomal maturation protein- via epididymosomes in the domestic cat model (Rowlison et al., 2017). Cenexin has been reported to act as a scaffold protein in somatic cell centrosomes to allow further binding of other proteins including ninein and centriolin (Ishikawa et al., 2005). The proteins, ninein, and centriolin have also been reported to aid in the maturation and function of the somatic cell centrosome (Delghyr et al., 2005, Ishikawa et al., 2005). Specifically, ninein serves in the nucleation and anchoring of microtubules, and centriolin aids in the formation of the centrosome during early cellular development. We also demonstrated the presence of these proteins with the sperm centrosome in the domestic cat (Rowlison et al., 2017). Epididymosome-exposed sperm cells in this study bordered on significantly increased proportions of penetrated oocytes with pronuclei that were either adjacent or had achieved fusion, possibly indicating improved centrosomal function. Additional replicates of these analyses will be completed to further discern differences in the fertilizing ability of epididymosome-exposed spermatozoa as compared to the control spermatozoa incubated in base media. Analyses to compare the size of the aster formed will also be completed to provide additional assessment of the centrosome function.

While many abnormalities of the sperm cell have been extensively studied, complications with the centrosome are not clearly understood in any felid species studied to date. Understanding the mechanisms of infertility is crucial to the development of assisted reproductive techniques that may assist in the breeding of an endangered species. Thus, one of the objectives of this study was to assess the presence of centrosome abnormalities in the domestic cat as a model for wild felids and determine the impact that these abnormalities may have on fertilization. This study is the first to demonstrate morphological abnormalities at the location of the centrosome, the head-tail junction in the domestic cat. As Chemes et al. (1999) demonstrated in the human, complications of the centrosome can be detected by assessing the morphology of at this location. Likewise, we observed morphological abnormalities including dissociated or misaligned heads, abnormally thin or thickened head-tail junctions, and fractured or discontinuous membranes. Males that were assessed as teratospermic via overall cell morphology were significantly more likely to exhibit abnormalities specifically at the head-tail junction. It is possible that the mechanisms which contribute to abnormalities at other locations of the sperm cell may also contribute to complications with the formation of the centrosome; however, this remains to be investigated in any species.

The success of oocyte penetration tended to decrease when co-incubating oocytes with spermatozoa of teratospermic males. Previous reports have demonstrated a diminished ability of teratospermic male samples to bind

with the zona pellucida and penetrate the oocyte (Howard et al., 1991 and 1993). This was observed in domestic cat oocytes with intact zona pellucida as well as zona-free hamster oocytes. Additional replicates of these analyses will be completed to further discern differences between normospermic and teratospermic individuals.

Overall, there was a very limited difference in the centrosome function of teratospermic males as compared to normospermic when all individuals were assessed as a whole. The majority of individuals (83.3% of the males assessed) displayed only a small proportion of sperm cells with abnormalities at the head-tail junction regardless of whether they were normospermic or teratospermic. *In vitro* analyses using spermatozoa isolated from these males resulted in oocytes with pronuclei that were adjacent or fused, indicating competent production of the sperm aster and the ability achieve pronuclear fusion. However, fertilization with a male that displayed 22% abnormal spermatozoa resulted in a 20% decrease in the proportion of oocytes displaying fused or adjacent pronuclei. Similarly, a study conducted by Chemes et al. (1999) assessed centrosomal function in sterile men and observed sperm samples with 25% abnormalities at the head-tail junction which subsequently resulted in decreased embryonic survivability following sperm injection. It is possible that the incidence of centrosome-related complications may only arise when an individual's sperm sample contains a certain minimal threshold of abnormal spermatozoa. Additional replicates of these analyses will be completed specifically using individuals with ≥20% head-tail junction abnormalities to

further discern the impact on pronuclear fusion. Determining this threshold would assist in the diagnosis of this potential source of infertility. Additionally, Chemes et al. (1999) observed the abnormal centrosome phenotype in two brothers, indicating a genetic origin of this syndrome. This further supports the need to establish effective methods of diagnosis as this complication may not just be present in a single individual, but be genetically inherited by subsequent generations. This will also aid in determining if the use of assisted reproductive techniques may be required to overcome this source of infertility.

To date, very few studies have focused on centrosome-related abnormalities prior to fertilization. Chemes et al. (1999) attempted to isolate normal spermatozoa from a sterile man using a series of washes and centrifugation but did not have success in attaining better quality samples. Rawe et al. (2002) attempted sperm injection following the careful selection of spermatozoa that appeared normal in the collection from a sterile man. This resulted in increased fertilization and first cleavage, but still resulted in subsequent embryonic death. Thus, it is still uncertain how to successfully treat this type of infertility. Improving our understanding of the centrosome maturation process may contribute to the development of assisted reproductive techniques. Future studies in our lab will explore the use of incorporating epididymosomes in the medium used for *in vitro* culture systems prior to fertilization to assess whether there may be a beneficial effect on centrosomal function. It may be possible that individuals displaying this source of infertility are defective in the epididymal secretion of essential proteins that aid in the

proper maturation of the centrosome; therefore, exposing these sperm samples to epididymosomes isolated from normospermic males may allow for the acquisition of essential factors and improve the centrosomal function following oocyte penetration.

This study provides background knowledge of the sperm maturation process and the role of epididymosomes on the acquisition of functionality. It also provides further evidence of centrosome-related abnormalities in the domestic cat and the subsequent effect on fertilization. Together, this information will aid in the development of assisted reproductive techniques and improve the treatment of centrosomal-related infertility.

Conclusion

This study demonstrated a functional effect of epididymosome exposure on the acquisition of functionality, specifically the ability to penetrate an oocyte, produce a sperm aster, and undergo pronuclear fusion. Furthermore, this study also demonstrated that the domestic cat displays morphological abnormalities at the location of the centrosome, the head-tail junction, with diminished centrosome functionality following fertilization when using a sperm sample with increased head-tail junction abnormalities. These results improve our understanding of the underlying mechanisms of the sperm cell maturation process as well as centrosomal-related complications and may contribute to the

future development of assisted reproductive techniques for many species, including endangered felids.

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References

Caballero, J. N., Frenette, G., Belleannée, C., & Sullivan, R. (2013). CD9-positive microvesicles mediate the transfer of molecules to bovine spermatozoa during epididymal maturation. PloS one, 8(6), e65364.

Chemes, H. E., Puigdomenech, E. T., Carizza, C., Olmedo, S. B., Zanchetti, F., & Hermes, R. (1999). Acephalic spermatozoa and abnormal development of the head neck attachment: a human syndrome of genetic origin. Human reproduction, 14(7): 1811-1818.

Comizzoli, P., Wildt, D. E., & Pukazhenthi, B. S. (2006). Poor centrosomal function of cat testicular spermatozoa impairs embryo development in vitro after intracytoplasmic sperm injection. Biology of reproduction, 75(2), 252-260.

Dacheux, J. L., & Dacheux, F. (2014). New insights into epididymal function in relation to sperm maturation. Reproduction, 147(2), R27-R42.

Delgehyr, N., Sillibourne, J., & Bornens, M. (2005). Microtubule nucleation and anchoring at the centrosome are independent processes linked by ninein function. Journal of cell science, 118(8), 1565-1575.

Fornes, M. W., Barbieri, A., & Cavicchia, J. C. (1995). Morphological and enzymatic study of membrane-bound vesicles from the lumen of the rat epididymis. Andrologia, 27(1), 1-5.

Frenette, G., & Sullivan, R. (2001). Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. Molecular reproduction and development, 59(1), 115-121.

Hermo, L., & Jacks, D. (2002). Nature's ingenuity: bypassing the classical secretory route via apocrine secretion. Molecular reproduction and development, 63(3), 394-410.

Howard, J., Bush, M., & Wildt, D. E. (1991). Teratospermia in Domestic Cats Compromises Penetration of Zona-Free Hamster Ova and Cat Zonae Pellucidae. Journal of andrology, 12(1), 36-45.

Howard, J. G., Donoghue, A. M., Johnston, L. A., & Wildt, D. E. (1993). Zona pellucida filtration of structurally abnormal spermatozoa and reduced fertilization in teratospermic cats. Biology of reproduction, 49(1), 131-139.

Ishikawa H, K. A. T. S. T. S. (2005). Odf2-deficient mother centrioles lack distal/subdistal appendages and the ability to generate primary cilia. Nature cell biology, 7(5): 517-24.

IUCN Red List of Threatened Species. Version 2017.1. <http://www.iucnredlist.org>

Légaré, C., Bérubé, B., Boué, F., Lefièvre, L., Morales, C. R., El-Alfy, M., & Sullivan, R. (1999). Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein. Molecular reproduction and development, 52(2), 225-233.

Morales, A., & Cavicchia, J. C. (1991). Release of cytoplasmic apical protrusions from principal cells of the cat epididymis, an electron microscopic study. Tissue and cell, 23(4), 505-513.

Navara, C. S., First, N. L., & Schatten, G. (1996). Phenotypic variations among paternal centrosomes expressed within the zygote as disparate microtubule lengths and sperm aster organization: correlations between centrosome activity and developmental success. Proceedings of the National Academy of Sciences, 93(11), 5384-5388.

Pukazhenthi, B. S., Neubauer, K., Jewgenow, K., Howard, J., & Wildt, D. E. (2006). The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology, 66(1), 112-121.

Rawe, V. Y., Terada, Y., Nakamura, S., Chillik, C. F., Olmedo, S. B., & Chemes, H. E. (2002). A pathology of the sperm centriole responsible for defective sperm aster formation, syngamy and cleavage. Human Reproduction, 17(9), 2344-2349.

Rejraji, H., Vernet, P., & Drevet, J. R. (2002). GPX5 is present in the mouse caput and cauda epididymidis lumen at three different locations. Molecular reproduction and development, 63(1), 96-103.

Rowlison, T., Ottinger, M. A., & Comizzoli, P. (2017). Deciphering the mechanisms involving cenexin, ninein and centriolin in sperm maturation during epididymal transit in the domestic cat. Reproduction in domestic animals, 52(S2), 193-196.

Rowlison, T. Ottinger, M.A., Comizzoli, P. Key factors enhancing sperm fertilizing ability are transferred from the epididymis to the spermatozoa via epididymosomes in the domestic cat model. Biology of reproduction, Submitted.

Saez, F., Frenette, G., & Sullivan, R. (2003). Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. Journal of andrology, 24(2), 149-154.

Schatten, G. (1994). The Centrosome and Its Mode of Inheritance: The Reduction of the Centrosome during Gametogenesis and Its Restoration during Fertilization. Developmental biology, 165(2): 299-335.

Sullivan, R. (1999). Interaction between sperm and epididymal secretory proteins. The male gamete: from basic to clinical applications, 130-136.

Sullivan, R., & Saez, F. (2013). Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. Reproduction, 146(1), R21-R35.

Thimon, V., Koukoui, O., Calvo, E., & Sullivan, R. (2007). Region-specific gene expression profiling along the human epididymis. Molecular human reproduction, 13(10), 691-704.

Chapter 5: Discussion.

The goal of this research was to explore key phases in the sperm maturation process associated with functional effects of protein incorporation via epididymosome secretion on sperm cell motility and fertilization. Understanding these physiological processes which lead to structurally and functionally mature spermatozoa is critical for overcoming common problems with infertility or developing efficient contraception strategies. Additionally, this research examined abnormalities of the centrosome and subsequent effects on the ability form a competent sperm aster and complete pronuclear fusion. Overall objectives of this research were to (1) identify essential proteins that contribute to sperm centrosome maturation throughout epididymal transit, (2) characterize the transfer of key factors via epididymosomes between the epididymis and maturing sperm cells and (3) demonstrate the impact of such mechanisms on the acquisition of motility and fertilizing ability by the spermatozoa. These studies were completed using the domestic cat (*Felis catus*) as it is a valuable biomedical model for human infertility and close relative to wild cat species (Comizzoli et al., 2008; Comizzoli and Wildt, 2012; Wildt et al., 2010).

Three essential proteins (cenexin, ninein, and centriolin) were successfully identified to associate with the centrosome, and the timing of incorporation by the sperm population was characterized throughout epididymal maturation. As these proteins have been observed to aid in the

centrosome function of somatic cells, their incorporation by the sperm cell centrosome indicate a potential role in the formation of the sperm aster formation following oocyte penetration (Ishikawa et al., 2005). Specifically, cenexin- a key protein involved in the centrosomal function and a maturational marker for this organelle, was observed to localize to the sperm cell population in a significantly increasing manner throughout maturation.

Localization of cenexin synthesis and secretion was next determined throughout the male reproductive tract. Cenexin was successfully detected within the tissue of each segment and its secretion observed throughout the epididymis. Transfer of this protein was also observed following exposure to isolated epididymal luminal fluid and epididymosomes, thus providing a method in which this essential protein can be incorporated by the sperm cell. The beneficial effect of epididymosome exposure on immature sperm cell motility was also demonstrated throughout incubation with observations of significantly increased percent motility and forward progressive movement as compared to spermatozoa incubated in base culture medium. The effect of epididymosome exposure on the ability form a competent sperm aster was also assessed with results demonstrating an increasing trend in the ability to achieve pronuclear fusion following oocyte penetration. Together, these analyses aid in our understanding of the sperm maturation process and provide background knowledge as to how essential proteins may be supplied to the sperm cell for the achievement of functionality.

Additionally, this research assessed the presence of centrosomal abnormalities in the domestic cat with multiple morphological anomalies observed. Further analyses demonstrated decreased rates of pronuclear fusion when using a sperm sample of increased centrosomal abnormalities, suggesting diminished functionality. Together, these results demonstrate that centrosomal abnormalities exist in the domestic cat species and may hinder fertility as has been observed in other species including the human (*Homo sapiens*) and bovine (*Bos taurus*; Chemes et al., 1999; Rawe et al., 2002).

Sperm maturation throughout epididymal transit

The information gained from this dissertation aids in our understanding of the maturation process during epididymal transit and helps bridge the gap between research efforts which focus on the development of sperm cells, such as studies focused on spermatogonial stem cells and testicular tissue xenografting (Kim et al., 2006; Vansandt et al., 2016; Snedaker et al., 2004; Kim et al., 2007), and the use of fully matured spermatozoa such as in the case of artificial insemination, *in vitro* fertilization, and subsequent analyses of embryonic development (Platz et al., 1978; Axnér et al., 2002; Tsutsui et al., 2000 and 2003; Johnston et al., 1989; Lengwinat et al., 1994; Spindler and Wildt, 1999; Karja, 2002). Efforts to examine the changes that occur in the sperm cell during epididymal transit have been ongoing for many years;

however, much remains unknown (Senger, 2005; Cooper 2012; Dacheux and Dacheux, 2014).

This dissertation demonstrated the beneficial role of epididymosomes on sperm acquisition of key proteins; specifically, using cenexin as a maturation marker, we demonstrated that exposure to epididymosomes resulted in the incorporation of this protein in a manner which was observed in spermatozoa that were matured *in vivo* (Chapter 1, Rowlison et al., 2017). The focus on cenexin for this dissertation was particularly informative as it is not only serves as a functional protein for the centrosome but indicates the overall maturation status of the cell.

Previous reports have demonstrated that similar structures produced by the prostate, termed "prostasomes", improve sperm motility, and ability to undergo capacitation (as reviewed by Frenette and Sullivan, 2003). However, exposure to prostasomes does not occur until ejaculation, when the sperm cells are introduced to the seminal fluid. As sperm cells remain in a quiescent state throughout epididymal transit, it is relatively difficult to determine the mechanisms involved in the acquisition of motility until time of ejaculation or when the spermatozoa are exposed to capacitating factors *in vitro* (Bork et al., 1988; Chevrier and Dacheus, 1992). The information provided by this dissertation provides evidence that epididymosomes aid in this functional acquisition during the time of epididymal transit. And while previous reports have assessed the presence of proteins which aid in the zona pellucida binding

and fertilization process in other species, the studies conducted in this dissertation are the first to directly demonstrate the beneficial effects of epididymosomes on fertilization success (Figure 5.1).

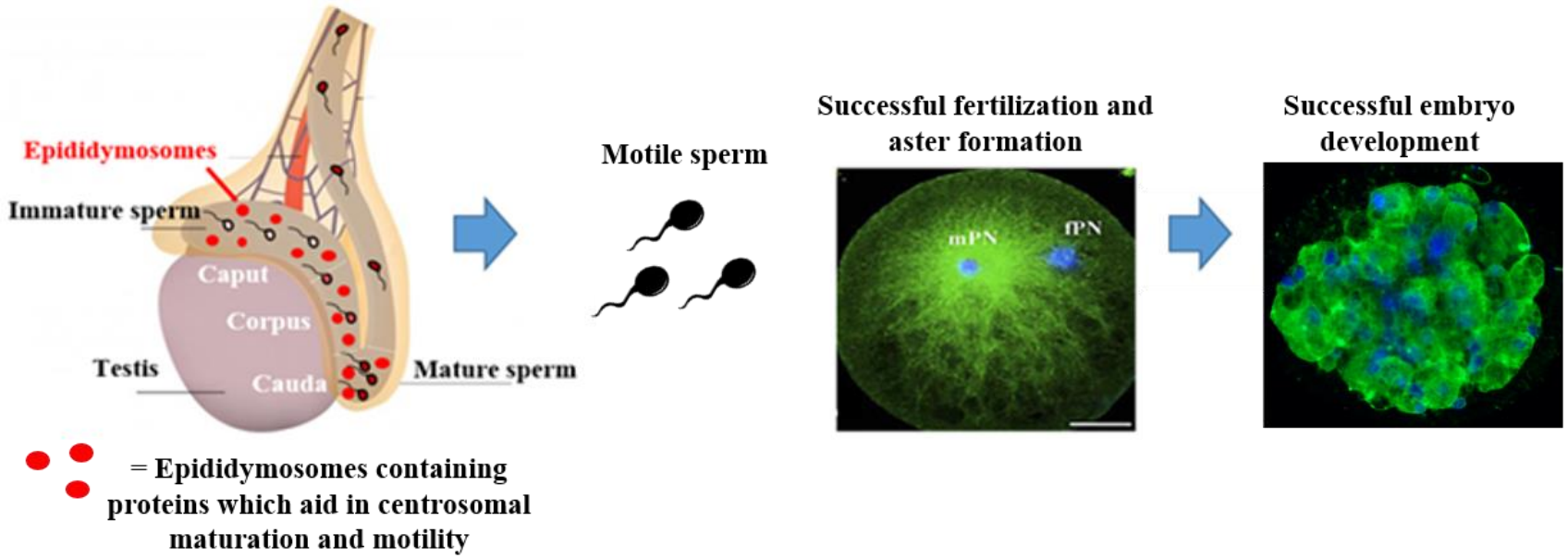

Figure 5.1. Working Hypothesis. Epididymosomes secreted throughout the epididymis contain key proteins which aid in centrosomal maturation and the acquisition of motility and fertilizing ability.

Further proteomic analyses are currently underway in our laboratory to determine the composition of these epididymosomes. These analyses may identify other key proteins that aid in sperm cell maturation which may have not been detected before due to potential limits in assessing the contents of epididymosomes. Identifying the protein content and physiological role of these vesicles will not only assist in the characterization of essential proteins that are necessary for sperm maturation but also emphasizes the importance of elucidating how these factors are supplied to the spermatozoa within the luminal fluid. Furthermore, these studies were carried out in the domestic cat and thus provide unique insight of a species which is more genetically related and a better representative of wild felids as compared to other commonly used animal models. While studies of epididymosome proteins have been completed in other species including the cow, human, stallion, rat, and hamster, (Girouard et al., 2011; Dacheux et al., 2006; Fouche´cour t et al., 2000; Fornés et al., 1995; Légaré et al., 1999), use of the domestic cat for this dissertation provides distinct information that more accurately represents the proteome and reproductive physiology of wild cat species.

Although many sperm abnormalities have been extensively studied, such as head and flagellum morphological abnormalities or impaired motility, complications with the sperm centrosome are not fully understood in any species studied to date (Pukazhenthi et al., 2006; Schatten, 1994; Navara et al., 1996; Chemes et al., 1999; Rawe et al., 2002). This research demonstrates that

while the centrosomal structure is first formed during the initial stages of spermatogenesis, complete maturation and acquisition of functionality is attained via secretion of cenexin, and possibly other proteins, that are supplied by the epididymal epithelium. This aids in bridging the gap of knowledge of studies that examine sperm cell ability to penetrate the zona pellucida and achieve subsequent embryonic cleavage (Howard et al., 1991; Johnston et al., 1989; Lengwinat et al., 1994; Spindler and Wildt, 1999; Karja, 2002). Specifically, this dissertation provides information demonstrating the essential process epididymal maturation on centrosomal functionality to achieve pronuclear fusion (Figure 5.1).

Potential application toward assisted reproductive technologies

This aim of this dissertation was to enhance our understanding of the sperm maturation process to improve the use of assisted reproductive techniques. The observed beneficial effect of epididymosomes on the on the sperm cell acquisition of motility and ability to fertilize an oocyte may indicate the potential use of isolating these vesicles for various assisted reproductive techniques. For example, the incorporation of epididymosomes into an *in vitro* culture system may improve the functionality of immature sperm cells isolated from the testis segment which may be the only source of gametes that are attainable from certain individuals. This may be applicable to certain situations such as collecting samples from juveniles which have not yet undergone sexual

maturation but must undergo medical treatments which render them infertile, such as chemotherapy, or sexually mature individuals that display certain types of infertility in which sperm maturation is not naturally achievable.

Even if it may not be feasible to isolate epididymosomes from a certain individual or species, assessing which key factors are supplied via this secretion mechanism can provide background information to modify the culture system to better mimic the natural maturation process of the sperm cell during epididymal transit. Creating a better culture system may also enhance other assisted reproductive techniques such as in the case of sperm cell cryopreservation. While this has become a vital tool for human infertility and in the conservation of endangered species, the freezing and thawing methods currently used have been observed to be detrimental to the sperm structure and function (Comizzoli et al., 2012). Exposing sperm cells after thawing to epididymosomes may assist in replenishing key proteins that may have been damaged or lost during the freezing process and restore sperm functionality. Plans to assess this potential beneficial effect in our lab are currently underway.

Information gained from this research will also aid in assessing complications related to abnormal centrosomal development and advance the use of assisted reproductive techniques to overcome this type of infertility. For example, exposing the spermatozoa of an infertile male to the key proteins and factors identified from these studies via an *in vitro* culture system may help artificially complete the centrosome's maturation. Alternatively, Comizzoli et al. (2006) demonstrated that the centrosome of a sperm cell may be replaced

with that of another individual. This provides another possible technique that may be used to treat infertile individuals such that the sperm head of an infertile individual may be injected into an oocyte, but with the centrosome of another fertile individual with a competent centrosome. This would allow for the genetic material of the infertile individual to be inherited while also restoring the ability to subsequently produce the aster and achieve embryonic development. While this is a possibility, it may be more feasible to instead use only the spermatozoa available from the infertile individual due to limitations in the equipment available or concerns of transmitting infectious agents from the sperm centrosome donors. For this reason, it is ever more critical that the centrosomal maturation process be understood so that the development of other more feasible techniques, such as *in vitro* culture, may be created and made available.

Development of contraceptive techniques

In addition to the potential application toward assisted reproductive techniques, information gained from this dissertation may also be applied toward developing contraceptive technologies. Creating effective contraception is essential for managing overly abundant wildlife populations as well as captive breeding populations (Pukazhenthi et al., 2005). This dissertation demonstrated a beneficial role of epididymosomes on the acquisition of sperm cell functionality. Thus, targeting these vesicles in a manner which inhibits the secretion or transport to the maturing sperm population within the epididymis may inhibit the development of the spermatozoa and render the individual

infertile. Likewise, the sperm centrosome may also be a potential target for contraceptive techniques as hindering the maturation of this organelle would also have a negative effect on the success of fertilization. While investigation into these potential approaches would be required to assess success and feasibility, the data presented in this dissertation provides valuable background information which may aid in the development of these techniques.

References

Axnér, E. (2006). Sperm maturation in the domestic cat. Theriogenology, 66(1), 14-24.

Bork, K., Chevrier, C., Paquignon, M., Jouannet, P., & Dacheux, J. L. (1988). Flagellar motility and movement of boar spermatozoa during epididymal transit. Reproduction, nutrition, developement, 28(5), 1307-1315.

Chemes, H. E., Puigdomenech, E. T., Carizza, C., Olmedo, S. B., Zanchetti, F., & Hermes, R. (1999). Acephalic spermatozoa and abnormal development of the head–neck attachment: a human syndrome of genetic origin. Human reproduction, 14(7), 1811-1818.

Chevrier, C., & Dacheux, J. L. (1992). Evolution of the flagellar waveform of ram spermatozoa in relation to the degree of epididymal maturation. Cytoskeleton, 23(1), 8-18.

Comizzoli, P., Songsasen, N., Hagedorn, M., & Wildt, D. E. (2012). Comparative cryobiological traits and requirements for gametes and gonadal tissues collected from wildlife species. Theriogenology, 78(8), 1666-1681.

Comizzoli, P., Wildt, D. E., & Pukazhenthi, B. S. (2006). Poor centrosomal function of cat testicular spermatozoa impairs embryo development in vitro after intracytoplasmic sperm injection. Biology of reproduction, 75(2), 252-260.

Cooper, T. G. (1998). Interactions between epididymal secretions and spermatozoa. Journal of reproduction and fertility. Supplement, 53, 119-136.

Dacheux, J.-L. and F.o. Dacheux. (2014). New insights into epididymal function in relation to sperm maturation. Reproduction, 147(2): p. R27-R42.

Fornes, M., A. Barbieri, and J. Cavicchia. (1995). Morphological and enzymatic study of membrane-bound vesicles from the lumen of the rat epididymis. Andrologia, 1995. 27(1): p. 1-5.

Fouchecourt, S., Sonia Métayer, S., Locatelli, A., Dacheux F., Dacheux, J. (2000). Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins. Biology of reproduction, 62(6): p. 1790-1803.

Girouard, J., Frenette, G., & Sullivan, R. (2011). Comparative proteome and lipid profiles of bovine epididymosomes collected in the intraluminal compartment of the caput and cauda epididymidis. International journal of andrology, 34(5pt2).

Hinchcliffe, E. H., Miller, F. J., Cham, M., Khodjakov, A., & Sluder, G. (2001). Requirement of a centrosomal activity for cell cycle progression through G1 into S phase. Science, 291(5508), 1547-1550.

Howard, J.G., Brown, J.L., Bush, M., & Wildt, D.E. (1990). Teratospermic and Normospermic Domestic Cats: Ejaculate Traits, Pituitary—Gonadal Hormones, and Improvement of Spermatozoal Motility and Morphology After Swim-Up Processing. Journal of andrology, 11(3), 204-215.

Howard, J., Bush, M., & Wildt, D. E. (1991). Teratospermia in Domestic Cats Compromises Penetration of Zona-Free Hamster Ova and Cat Zonae Pellucidae. Journal of andrology, 12(1), 36-45.

Ishikawa H, K. A. T. S. T. S. (2005). Odf2-deficient mother centrioles lack distal/subdistal appendages and the ability to generate primary cilia. Nature cell biology, 7(5): 517-24.

Johnston, L. A., O'brien, S. J., & Wildt, D. E. (1989). In vitro maturation and fertilization of domestic cat follicular oocytes. Molecular reproduction and development, 24(3), 343-356.

Karja, N. W. K., Otoi, T., Murakami, M., Fahrudin, M., & Suzuki, T. (2002). In vitro maturation, fertilization and development of domestic cat oocytes recovered from ovaries collected at three stages of the reproductive cycle. Theriogenology, 57(9), 2289-2298.

Khodjakov, A., Cole, R. W., Oakley, B. R., & Rieder, C. L. (2000). Centrosome-independent mitotic spindle formation in vertebrates. Current biology, 10(2), 59-67.

Kim, Y., Selvaraj, V., Dobrinski, I., Lee, H., McEntee, M. C., & Travis, A. J. (2006). Recipient preparation and mixed germ cell isolation for spermatogonial stem cell transplantation in domestic cats. Journal of andrology, 27(2), 248-256.

Kim, Y., Selvaraj, V., Pukazhenthi, B., & Travis, A. J. (2007). Effect of donor age on success of spermatogenesis in feline testis xenografts. Reproduction, fertility and development, 19(7), 869-876.

Légaré, C., Bérubé, B., Boué, F., Lefièvre, L., Morales, C. R., El-Alfy, M., & Sullivan, R. (1999). Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein. Molecular reproduction and development, 52(2), 225-233.

Lengwinat, T., & Blottner, S. (1994). In vitro fertilization of follicular oocytes of domestic cat using fresh and cryopreserved epididymal spermatozoa. Animal reproduction science, 35(3-4), 291-301.

Lingle, W. L., Lutz, W. H., Ingle, J. N., Maihle, N. J., & Salisbury, J. L. (1998). Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. Proceedings of the National Academy of Sciences, 95(6), 2950-2955.

Long, J. A., Wildt, D. E., Wolfe, B. A., Critser, J. K., DeRossi, R. V., & Howard, J. (1996). Sperm capacitation and the acrosome reaction are compromised in teratospermic domestic cats. Biology of reproduction, 54(3), 638-646.

Manandhar, G., Schatten, H., & Sutovsky, P. (2005). Centrosome reduction during gametogenesis and its significance. Biology of reproduction, 72(1), 2-13.

Navara, C., N. First, and G. Schatten. (1996). Phenotypic variations among paternal centrosomes expressed within the zygote as disparate microtubule lengths and sperm aster organization: correlations between centrosome activity and developmental success. Proceedings of the National Academy of Sciences, 93(11): p. 5384-5388.

Pihan, G. A., Purohit, A., Wallace, J., Knecht, H., Woda, B., Quesenberry, P., & Doxsey, S. J. (1998). Centrosome defects and genetic instability in malignant tumors. Cancer research, 58(17), 3974-3985.

Platz, C. C., Wildt, D. E., & Seager, S. W. J. (1978). Pregnancy in the domestic cat after artificial insemination with previously frozen spermatozoa. Journal of reproduction and fertility, 52(2), 279-282.

Pukazhenthi, B., Comizzoli, P., Travis, A. J., & Wildt, D. E. (2005). Applications of emerging technologies to the study and conservation of threatened and endangered species. Reproduction, fertility and development, 18(2), 77-90.

Pukazhenthi, B. S., Neubauer, K., Jewgenow, K., Howard, J., & Wildt, D. E. (2006). The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology, 66(1), 112-121.

Rawe, V. Y., Terada, Y., Nakamura, S., Chillik, C. F., Olmedo, S. B., & Chemes, H. E. (2002). A pathology of the sperm centriole responsible for defective sperm aster formation, syngamy and cleavage. Human reproduction, 17(9), 2344-2349.

Robbins, E., Jentzsch, G., & Micali, A. (1968). The centriole cycle in synchronized HeLa cells. The Journal of cell biology, 36(2), 329-339.

Rowlison, T., Ottinger, M. A., & Comizzoli, P. (2017). Deciphering the mechanisms involving cenexin, ninein and centriolin in sperm maturation during epididymal transit in the domestic cat. Reproduction in domestic animals, 52(S2), 193-196.

Schatten, G. (1994). The Centrosome and Its Mode of Inheritance: The Reduction of the Centrosome during Gametogenesis and Its Restoration during Fertilization. Developmental biology, 165(2): 299-335.

Senger, P.L. (2005). Pathways to pregnancy and parturition. 2 ed. Current Conceptions, Inc., 1615 NE Eastgate Blvd.

Snedaker, A. K., Honaramooz, A., & Dobrinski, I. (2004). A game of cat and mouse: xenografting of testis tissue from domestic kittens results in complete cat spermatogenesis in a mouse host. Journal of Andrology, 25(6), 926-930.

Spindler, R. E., & Wildt, D. E. (1999). Circannual variations in intraovarian oocyte but not epididymal sperm quality in the domestic cat. Biology of reproduction, 61(1), 188-194.

Tsutsui, T., Tanaka, A., Takagi, Y., Nakagawa, K., Fujimoto, Y., Murai, M., Anzai, M., and Hori, T. (2000). Unilateral intrauterine horn insemination of frozen semen in cats. Journal of veterinary medical science, 62(12), 1247-1251

Tsutsui, T., Wada, M., Anzai, M., and Hori, T. (2003). Artificial insemination with frozen epididymal sperm in cats. Journal of veterinary medical science, 65, no. 3: 397-399.

Vansandt, L. M., Livesay, J. L., Dickson, M. J., Li, L., Pukazhenthi, B. S., & Keefer, C. L. (2016). Conservation of spermatogonial stem cell marker expression in undifferentiated felid spermatogonia. Theriogenology, 86(4), 1022-1035.

Vorobjev, I. A., & YuS, C. (1982). Centrioles in the cell cycle. I. Epithelial cells. The Journal of cell biology, 93(3), 938-949

Wildt, D. E., Comizzoli, P., Pukazhenthi, B., & Songsasen, N. (2010). Lessons from biodiversity—the value of nontraditional species to advance reproductive science, conservation, and human health. Molecular reproduction and development, 77(5), 397-409.

Wildt, D. E., Ellis, S., & Howard, J. G. (2001). Linkage of reproductive sciences: from 'quick fix' to 'integrated' conservation. Journal of reproduction and fertility. Supplement, 57, 295-307.

Appendices

Appendix 1: Comprehensive Analysis of Sperm Cell Abnormalities

	Overall Morphology (%)		Abnormalities (%)									
Male	Normal	Abnormal	Classification	Large or small head	Multiple Heads	Missing Acrosome	Abnormal Centrosome	Bent Midpiece	Cytoplasmic Droplet	Coiled Tail	Bent Tail	Multiple Tails
1	97	3	Normospermic	0	0	0	3	0	0	2	1	0
2	43	57	Normospermic	0	0	3	3	8	2	43	2	0
3	73	27	Normospermic	2	1	3	0	4	0	17	0	0
4	41	59	Normospermic	1	0	9	8	5	3	48	2	0
5	45	55	Normospermic	0	1	2	1	1	1	51	0	0
6	26	74	Teratospermic	3	1	14	22	12	1	43	5	2
7	23	77	Teratospermic	0	0	10	3	5	1	65	0	0
8	15	85	Teratospermic	0	0	14	20	5	3	72	0	1
9	30	70	Teratospermic	3	0	16	10	3	0	51	1	0
10	33	67	Teratospermic	0	0	3	8	1	1	62	1	0
11	11	89	Teratospermic	2	0	2	3	1	3	79	2	0
12	19	81	Teratospermic	0	0	8	6	1	0	74	0	0

Compiled References

Askham, J. M., Vaughan, K. T., Goodson, H. V., & Morrison, E. E. (2002). Evidence that an interaction between EB1 and p150Glued is required for the formation and maintenance of a radial microtubule array anchored at the centrosome. Molecular biology of the cell, 13(10), 3627-3645.

Axnér, E. (2006). Sperm maturation in the domestic cat. Theriogenology, 66(1), 14-24.

Belleannée, C., Silva, N. D., Shum, W. W. C., Marsolais, M., Laprade, R., Brown, D., & Breton, S. (2009). Segmental expression of the bradykinin type 2 receptor in rat efferent ducts and epididymis and its role in the regulation of aquaporin 9. Biology of reproduction, 80(1), 134-143.

Bork, K., Chevrier, C., Paquignon, M., Jouannet, P., & Dacheux, J. L. (1988). Flagellar motility and movement of boar spermatozoa during epididymal transit. Reproduction, nutrition, developement, 28(5), 1307-1315.

Burfeind, P. and S. Hoyer-Fender. (1991). Sequence and developmental expression of a mRNA encoding a putative protein of rat sperm outer dense fibers. Developmental biology, 148(1): 195-204.

Caballero, J. N., Frenette, G., Belleannée, C., & Sullivan, R. (2013). CD9-positive microvesicles mediate the transfer of molecules to bovine spermatozoa during epididymal maturation. PloS one, 8(6), e65364.

Chemes, H. E., Puigdomenech, E. T., Carizza, C., Olmedo, S. B., Zanchetti, F., & Hermes, R. (1999). Acephalic spermatozoa and abnormal development of the head–neck attachment: a human syndrome of genetic origin. Human reproduction, 14(7), 1811-1818.

Chemes, H. E. (2012). Sperm centrioles and their dual role in flagellogenesis and cell cycle of the zygote. In The Centrosome (pp. 33-48). Humana Press.

Chen, Z., Indjeian, V. B., McManus, M., Wang, L., & Dynlacht, B. D. (2002). CP110, a cell cycle-dependent CDK substrate, regulates centrosome duplication in human cells. Developmental cell, 3(3), 339-350.

Chevrier, C., & Dacheux, J. L. (1992). Evolution of the flagellar waveform of ram spermatozoa in relation to the degree of epididymal maturation. Cytoskeleton, 23(1), 8-18.

Comizzoli, P., Songsasen, N., & Wildt, D. E. (2010). Protecting and extending fertility for females of wild and endangered mammals. In ocofertility, (pp. 87-100). Springer US.

Comizzoli, P., Wildt, D. E., & Pukazhenthi, B. S. (2006). Poor centrosomal function of cat testicular spermatozoa impairs embryo development in vitro after intracytoplasmic sperm injection. Biology of reproduction, 75(2), 252-260.

Comizzoli, P., & Wildt, D. E. (2012). On the horizon for fertility preservation in domestic and wild carnivores. Reproduction in domestic animals, 47(s6), 261-265.

Comizzoli, P., Wildt, D. E., & Pukazhenthi, B. S. (2004). Effect of 1, 2-propanediol versus 1, 2-ethanediol on subsequent oocyte maturation, spindle integrity, fertilization, and embryo development in vitro in the domestic cat. Biology of reproduction, 71(2), 598-604.

Comizzoli, P., Songsasen, N., Hagedorn, M., & Wildt, D. E. (2012). Comparative cryobiological traits and requirements for gametes and gonadal tissues collected from wildlife species. Theriogenology, 78(8), 1666-1681.

Comizzoli, P., Wildt, D. E., & Pukazhenthi, B. S. (2008). Impact of anisosmotic conditions on structural and functional integrity of cumulus–oocyte complexes at the germinal vesicle stage in the domestic cat. Molecular reproduction and development, 75(2), 345-354.

Cooper, T. G. (1998). Interactions between epididymal secretions and spermatozoa. Journal of reproduction and fertility. Supplement, 53, 119-136.

Crozet, N., Dahirel, M., & Chesne, P. (2000). Centrosome inheritance in sheep zygotes: centrioles are contributed by the sperm. Microscopy research and technique, 49(5), 445-450.

Dacheux, J. L., & Dacheux, F. (2002). Protein secretion in the epididymis. The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Publishers, 151-168.

Dacheux, J.L., J.L. Gatti, and F.o. Dacheux (2003). Contribution of epididymal secretory proteins for spermatozoa maturation. Microscopy research and technique, 61(1): p. 7-17.

Dacheux, J.-L. and F.o. Dacheux. (2014). New insights into epididymal function in relation to sperm maturation. Reproduction, 147(2): p. R27-R42.

Delgehyr, N., Sillibourne, J., & Bornens, M. (2005). Microtubule nucleation and anchoring at the centrosome are independent processes linked by ninein function. Journal of cell science, 118(8), 1565-1575.

D'Amours, O., Bordeleau, L. J., Frenette, G., Blondin, P., Leclerc, P., & Sullivan, R. (2012). Binder of sperm 1 and epididymal sperm binding protein 1 are associated with different bull sperm subpopulations. Reproduction, 143(6), 759-771.

Eickhoff, R., Wilhelm, B., Renneberg, H., Wennemuth, G., Bacher, M., Linder, D., Bucala, R., Seitz, J. and Meinhardt, A. (2001). Purification and characterization of macrophage migration inhibitory factor as a secretory protein from rat epididymis: evidences for alternative release and transfer to spermatozoa. Molecular medicine, 7(1), 27.

Elliott, G. D., Lee, P. C., Paramore, E., Van Vorst, M., & Comizzoli, P. (2015). Resilience of oocyte germinal vesicles to microwave-assisted drying in the domestic cat model. Biopreservation and biobanking, 13(3), 164-171.

Fawcett, D. W. (1975). The mammalian spermatozoon. Developmental biology, 44(2): 394-436.

Fawcett, D. W. and S. Ito (1965). The fine structure of bat spermatozoa. American journal of anatomy, 116(3): 567-609.

Fornes, M., A. Barbieri, and J. Cavicchia. (1995). Morphological and enzymatic study of membrane-bound vesicles from the lumen of the rat epididymis. Andrologia, 27(1): p. 1-5.

Fouchecourt, S., Sonia Métayer, S., Locatelli, A., Dacheux F., Dacheux, J. (2000). Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins. Biology of reproduction, 62(6): p. 1790-1803.

Frenette, G., Lessard, C., Madore, E., Fortier, M. A., & Sullivan, R. (2003). Aldose reductase and macrophage migration inhibitory factor are associated with epididymosomes and spermatozoa in the bovine epididymis. Biology of reproduction, 69(5), 1586-1592.

Frenette, G., C. Lessard, and R. Sullivan. (2002). Selected proteins of "prostasome-like particles" from epididymal cauda fluid are transferred to epididymal caput spermatozoa in bull. Biology of reproduction, 67(1): p. 308-313.

Frenette, G. and R. Sullivan. (2001). Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. Molecular reproduction and development, 59(1): p. 115-121.

Gaglio, T., Saredi, A., & Compton, D. A. (1995). NuMA is required for the organization of microtubules into aster-like mitotic arrays. The Journal of cell biology, 131(3), 693-708.

Gagnon, C. (1999). The male gamete: From basic to clinical applications.

Gatti, J. L., Métayer, S., Belghazi, M., Dacheux, F., & Dacheux, J. L. (2005). Identification, proteomic profiling, and origin of ram epididymal fluid exosome-like vesicles. Biology of reproduction, 72(6), 1452-1465.

Girouard, J., Frenette, G., & Sullivan, R. (2011). Comparative proteome and lipid profiles of bovine epididymosomes collected in the intraluminal compartment of the caput and cauda epididymidis. International journal of andrology, 34(5pt2).

Gómez, M. C., Pope, E., Harris, R., Mikota, S., & Dresser, B. L. (2003). Development of in vitro matured, in vitro fertilized domestic cat embryos following cryopreservation, culture and transfer. Theriogenology, 60(2), 239-251.

Goto, M., O'brien, D. A., & Eddy, E. M. (2010). Speriolin is a novel human and mouse sperm centrosome protein. Human reproduction, 25(8), 1884-1894.

Gould, M. C. and J. L. Stephano (1999). MAP Kinase, Meiosis, and Sperm Centrosome Suppression in Urechis caupo. Developmental biology, 216(1): 348-358.

Hermo, L. and D. Jacks. (2002). Nature's ingenuity: bypassing the classical secretory route via apocrine secretion. Molecular reproduction and development, 63(3): p. 394-410.

Hoyer-Fender, S. (2012). Centrosomes in fertilization, early embryonic development, stem cell division, and cancer.

Hüber, H.-F. (2007). Alternative splicing of exon 3b gives rise to ODF2 and Cenexin. Cytogenetic and genome research, 119(1-2): 68-73.

Howard, J., Bush, M., & Wildt, D. E. (1991). Teratospermia in Domestic Cats Compromises Penetration of Zona-Free Hamster Ova and Cat Zonae Pellucidae. Journal of andrology, 12(1), 36-45.

Howard, J. G., Donoghue, A. M., Johnston, L. A., & Wildt, D. E. (1993). Zona pellucida filtration of structurally abnormal spermatozoa and reduced fertilization in teratospermic cats. Biology of reproduction, 49(1), 131-139.

Ishikawa H, K. A. T. S. T. S. (2005). Odf2-deficient mother centrioles lack distal/subdistal appendages and the ability to generate primary cilia. Nature cell biology, 7(5): 517-24.

IUCN Red List of Threatened Species. Version 2017.1. <http://www.iucnredlist.org>

Jewgenow, K., Penfold, L. M., Meyer, H. H. D., & Wildt, D. E. (1998). Viability of small preantral ovarian follicles from domestic cats after cryoprotectant exposure and cryopreservation. Journal of reproduction and fertility, 112(1), 39-47.

Johnston, L. A., O'brien, S. J., & Wildt, D. E. (1989). In vitro maturation and fertilization of domestic cat follicular oocytes. Molecular Reproduction and development, 24(3), 343-356.

Karja, N. W. K., Otoi, T., Murakami, M., Fahrudin, M., & Suzuki, T. (2002). In vitro maturation, fertilization and development of domestic cat oocytes recovered from ovaries collected at three stages of the reproductive cycle. Theriogenology, 57(9), 2289-2298.

Kim, Y., Selvaraj, V., Dobrinski, I., Lee, H., McEntee, M. C., & Travis, A. J. (2006). Recipient preparation and mixed germ cell isolation for spermatogonial stem cell transplantation in domestic cats. Journal of andrology, 27(2), 248-256.

Kim, Y., Selvaraj, V., Pukazhenthi, B., & Travis, A. J. (2007). Effect of donor age on success of spermatogenesis in feline testis xenografts. Reproduction, fertility and development, 19(7), 869-876.

Kirchhoff, C., & Hale, G. (1996). Cell-to-cell transfer of glycosylphosphatidylinositol-anchored membrane proteins during sperm maturation. MHR: Basic science of reproductive medicine, 2(3), 177-184.

Kitiyanant, Y., Saikhun, J., & Pavasuthipaisit, K. (2003). Somatic cell nuclear transfer in domestic cat oocytes treated with IGF-I for in vitro maturation. Theriogenology, 59(8), 1775-1786.

Lange, B. M. and K. Gull. (1995). A molecular marker for centriole maturation in the mammalian cell cycle. The journal of cell biology, 130(4): 919-927.

Légaré, C., Bérubé, B., Boué, F., Lefièvre, L., Morales, C. R., El-Alfy, M., & Sullivan, R. (1999). Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein. Molecular reproduction and development, 52(2), 225-233.

Lengwinat, T., & Blottner, S. (1994). In vitro fertilization of follicular oocytes of domestic cat using fresh and cryopreserved epididymal spermatozoa. Animal reproduction science, 35(3-4), 291-301.

Luvoni, G. C., & Pellizzari, P. (2000). Embryo development in vitro of cat oocytes cryopreserved at different maturation stages. Theriogenology, 53(8), 1529-1540.

Luvoni, G. C., Pellizzari, P., & Battocchio, M. (1997). Effects of slow and ultrarapid freezing on morphology and resumption of meiosis in immature cat oocytes. Journal of reproduction and fertility. Supplement, 51, 93-98.

Martin-DeLeon, P. A. (2006). Epididymal SPAM1 and its impact on sperm function. Molecular and cellular endocrinology, 250(1), 114-121.

McNally, K. L., Fabritius, A. S., Ellefson, M. L., Flynn, J. R., Milan, J. A., & McNally, F. J. (2012). Kinesin-1 prevents capture of the oocyte meiotic spindle by the sperm aster. Developmental cell, 22(4), 788-798.

Morales, A. and J.C. Cavicchia. (1991). Release of cytoplasmic apical protrusions from principal cells of the cat epididymis, an electron microscopic study. Tissue and cell, 23(4): p. 505-513.

Mouttham, L., & Comizzoli, P. (2016). The preservation of vital functions in cat ovarian tissues during vitrification depends more on the temperature of the cryoprotectant exposure than on the sucrose supplementation. Cryobiology, 73(2), 187-195.

Murakami, M. K., Otoi, T., Karja, N. W. K., Wongsrikeao, P., Agung, B., & Suzuki, T. (2004). Blastocysts derived from in vitro-fertilized cat oocytes after vitrification and dilution with sucrose. Cryobiology, 48(3), 341-348.

Navara, C., N. First, and G. Schatten. (1996). Phenotypic variations among paternal centrosomes expressed within the zygote as disparate microtubule lengths and sperm aster organization: correlations between centrosome activity and developmental success. Proceedings of the National Academy of Sciences, 93(11): p. 5384-5388.

Neubauer, K., Jewgenow, K., Blottner, S., Wildt, D. E., & Pukazhenthi, B. S. (2004). Quantity rather than quality in teratospermic males: a histomorphometric and flow cytometric evaluation of spermatogenesis in the domestic cat (Felis catus). Biology of reproduction, 71(5), 1517-1524.

Nicander, L., & Malmqvist, M. (1977). Ultrastructural observations suggesting merocrine secretion in the initial segment of the mammalian epididymis. Cell and tissue research, 184(4), 487-490.

Ou, Y. Y., Mack, G. J., Zhang, M., & Rattner, J. B. (2002). CEP110 and ninein are located in a specific domain of the centrosome associated with centrosome maturation. Journal of cell science, 115(9), 1825-1835.

Palermo, G. D., Colombero, L. T., & Rosenwaks, Z. (1997). The human sperm centrosome is responsible for normal syngamy and early embryonic development. Reviews of reproduction, 2(1), 19-27.

Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature methods, 8(10), 785-786.

Platz, C. C., Wildt, D. E., & Seager, S. W. J. (1978). Pregnancy in the domestic cat after artificial insemination with previously frozen spermatozoa. Journal of reproduction and fertility, 52(2), 279-282.

Pope, C. E., Turner, J. L., Quatman, S. P., & Dresser, B. L. (1991). Semen storage in the domestic felid: a comparison of cryopreservation methods and storage temperatures. Biology of reproduction, 44(Suppl 1), 117.

Pukazhenthi, B., Comizzoli, P., Travis, A. J., & Wildt, D. E. (2005). Applications of emerging technologies to the study and conservation of threatened and endangered species. Reproduction, fertility and development, 18(2), 77-90.

Pukazhenthi, B. S., Neubauer, K., Jewgenow, K., Howard, J., & Wildt, D. E. (2006). The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology, 66(1), 112-121.

Pukazhenthi, B., Pelican, K., Wildt, D., & Howard, J. (1999). Sensitivity of domestic cat (Felis catus) sperm from normospermic versus teratospermic donors to cold-induced acrosomal damage. Biology of reproduction, 61(1), 135-141.

Pukazhenthi, B., Spindler, R., Wildt, D., Bush, L. M., & Howard, J. (2002). Osmotic properties of spermatozoa from felids producing different proportions of pleiomorphisms: influence of adding and removing cryoprotectant. Cryobiology, 44(3), 288-300.

Pukazhenthi, B. S., Wildt, D. E., & Howard, J. G. (2000). The phenomenon and significance of teratospermia in felids. Journal of reproduction and fertility. Supplement, 57, 423-433.

Rawe, V. Y., Terada, Y., Nakamura, S., Chillik, C. F., Olmedo, S. B., & Chemes, H. E. (2002). A pathology of the sperm centriole responsible for defective sperm aster formation, syngamy and cleavage. Human reproduction, 17(9), 2344-2349.

Rejraji, H., Vernet, P., & Drevet, J. R. (2002). GPX5 is present in the mouse caput and cauda epididymidis lumen at three different locations. Molecular reproduction and development, 63(1), 96-103.

Rowlison, T., Ottinger, M. A., & Comizzoli, P. (2017). Deciphering the mechanisms involving cenexin, ninein and centriolin in sperm maturation during epididymal transit in the domestic cat. Reproduction in domestic animals, 52(S2), 193-196.

Saez, F., Frenette, G., & Sullivan, R. (2003). Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. Journal of andrology, 24(2), 149-154.

Salmon, N. A., Reijo Pera, R. A., & Xu, E. Y. (2006). A gene trap knockout of the abundant sperm tail protein, outer dense fiber 2, results in preimplantation lethality. Genesis, 44(11), 515-522.

Sathananthan, A. H., Tatham, B., Dharmawardena, V., Grills, B., Lewis, I., & Trounson, A. (1997). Inheritance of sperm centrioles and centrosomes in bovine embryos. Archives of andrology, 38(1), 37-48.

Schatten, G. (1994). The Centrosome and Its Mode of Inheritance: The Reduction of the Centrosome during Gametogenesis and Its Restoration during Fertilization. Developmental biology, 165(2): 299-335.

Schatten, H. and Q.-Y. Sun (2009). The role of centrosomes in mammalian fertilization and its significance for ICSI. Molecular human reproduction, 15(9): 531-538.

Schmehl, M. and E. Graham (1989). Ultrastructure of the domestic tom cat (Felisdomestica) and tiger (Pantheratigrisaltaica) spermatozoa. Theriogenology, 31(4): 861-874.

Schmidt, T. I., Kleylein-Sohn, J., Westendorf, J., Le Clech, M., Lavoie, S. B., Stierhof, Y. D., & Nigg, E. A. (2009). Control of centriole length by CPAP and CP110. Current biology, 19(12), 1005-1011.

Senger, P.L. (2005). Pathways to pregnancy and parturition. 2 ed.: Current Conceptions, Inc., 1615 NE Eastgate Blvd.

Spektor, A., Tsang, W. Y., Khoo, D., & Dynlacht, B. D. (2007). Cep97 and CP110 suppress a cilia assembly program. Cell, 130(4), 678-690.

Spindler, R. E., & Wildt, D. E. (1999). Circannual variations in intraovarian oocyte but not epididymal sperm quality in the domestic cat. Biology of reproduction, 61(1), 188-194.

Snedaker, A. K., Honaramooz, A., & Dobrinski, I. (2004). A game of cat and mouse: xenografting of testis tissue from domestic kittens results in complete cat spermatogenesis in a mouse host. Journal of andrology, 25(6), 926-930.

Stearns T., and Kirschner M. (1994). In vitro reconstitution of centrosome assembly and function: the central role of γ-tubulin. Cell, 76.4, 623-637.

Stephano, J. L. and M. C. Gould. (2000). MAP kinase, a universal suppressor of sperm centrosomes during meiosis? Developmental biology, 222(2): 420-428.

Sullivan, R. (1999). Interaction between sperm and epididymal secretory proteins. The male gamete: from basic to clinical applications, 130-136.

Sullivan R, Frenette G, Girouard J. (2007). Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. Asian journal of andrology, 9:483–91.

Swanson, W. F., & Godke, R. A. (1994). Transcervical embryo transfer in the domestic cat. Laboratory animal science (USA).

Syntin, P., Dacheux, F., Druart, X., Gatti, J. L., Okamura, N., & Dacheux, J. L. (1996). Characterization and identification of proteins secreted in the various regions of the adult boar epididymis. Biology of reproduction, 55(5), 956-974.

Szebenyi, G., Hall, B., Yu, R., Hashim, A. I., & Krämer, H. (2007). Hook2 localizes to the centrosome, binds directly to centriolin/CEP110 and contributes to centrosomal function. Traffic, 8(1), 32-46.

Terrell, K.A., Wildt, D.E., Anthony, N.M., Bavister, B.D., Leibo, S.P., Penfold, L.M., Marker, L.L. and Crosier, A.E. (2011). Glycolytic enzyme activity is essential for domestic cat (Felis catus) and cheetah (Acinonyx jubatus) sperm motility and viability in a sugar-free medium. Biology of reproduction, 84(6), 1198-1206.

"Testis." 2015; Available from: http://www.britannica.com/EBchecked/topic/588769/testis.

Thimon, V., Koukoui, O., Calvo, E., & Sullivan, R. (2007). Region-specific gene expression profiling along the human epididymis. Molecular human reproduction, 13(10), 691-704.

Tsutsui, T., Tanaka, A., Takagi, Y., Nakagawa, K., Fujimoto, Y., Murai, M., Anzai, M., and Hori, T. (2000). Unilateral intrauterine horn insemination of frozen semen in cats. Journal of veterinary medical science, 62(12), 1247-1251

Tsutsui, T., Wada, M., Anzai, M., and Hori, T. (2003). Artificial insemination with frozen epididymal sperm in cats. Journal of veterinary medical science, 65, no. 3: 397-399.

Turner, R. M. (2005). Moving to the beat: a review of mammalian sperm motility regulation. Reproduction, fertility and development, 18(2), 25-38

Vansandt, L. M., Livesay, J. L., Dickson, M. J., Li, L., Pukazhenthi, B. S., & Keefer, C. L. (2016). Conservation of spermatogonial stem cell marker expression in undifferentiated felid spermatogonia. Theriogenology, 86(4), 1022-1035.

Wildt, D. E., Comizzoli, P., Pukazhenthi, B., & Songsasen, N. (2010). Lessons from biodiversity—the value of nontraditional species to advance reproductive science,

conservation, and human health. Molecular reproduction and development, 77(5), 397-409.

Wildt, D. E., Ellis, S., & Howard, J. G. (2001). Linkage of reproductive sciences: from 'quick fix' to 'integrated' conservation. Journal of reproduction and fertility. Supplement, 57, 295-307.

Wu, X., & Palazzo, R. E. (1999). Differential regulation of maternal vs. paternal centrosomes. Proceedings of the National Academy of Sciences, 96(4), 1397-1402.

Yin, X., Lee, Y., Lee, H., Kim, N., Kim, L., Shin, H., & Kong, I. (2006). In vitro production and initiation of pregnancies in inter-genus nuclear transfer embryos derived from leopard cat (Prionailurus bengalensis) nuclei fused with domestic cat (Felis silverstris catus) enucleated oocytes. Theriogenology, 66(2), 275-282.

Zambelli, D., Caneppele, B., Castagnetti, C., & Belluzzi, S. (2002). Cryopreservation of cat semen in straws: comparison of five different freezing rates. Reproduction in domestic animals, 37(5), 310-313.

Zhen, W., Li, P., He, B., Guo, J., & Zhang, Y. L. (2009). The novel epididymis-specific beta-galactosidase-like gene Glb1l4 is essential in epididymal development and sperm maturation in rats. Biology of reproduction, 80(4), 696-706.

Zamboni, L. and M. Stefanini (1971). The fine structure of the neck of mammalian spermatozoa. The anatomical record, 169(2): 155-172.

Zheng, Y., Wong, M. L., Alberts, B., & Mitchison, T. (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. Nature, 378(6557), 578.