SELECTION OF SEX-SPECIFIC APTAMER PROBES TO SPERM

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of

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by

ADAM JOSEPH COLLEY

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ABSTRACT

SELECTION OF SEX SPECIFIC APTAMER PROBES TO SPERM

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Sperm are haploid cells that carry either an X- or Y- sex chromosome, which upon fertilization ultimately determines sex in mammals. Other than this genetic difference, no clear molecular biomarker has been identified differentiating X-bearing sperm from Y-bearing sperm. This thesis is an investigation in the use of aptamer probes to resolve potential molecular differences between X- and Y- bull sperm. A method for the selection of aptamers to sperm was developed and applied here to the selection of sex-specific aptamers to sperm. The developed X- and Y-aptamers were found to have increased binding to X- and Y-sorted sperm, respectively. The aptamer sequences have been characterized to identify key features of X- and Y-sperm specific aptamers. A method for sex-typing single sperm has also been developed and applied to testing the sex-specificity of candidate aptamers. Aptamers developed herein would be useful for the development of novel sex-selection methods in livestock.

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LIST OF ABBREVIATIONS

(alphabetical order)

ACDU	automated cell disposition unit
AKAP4	A-kinase anchoring protein 4 (gene symbol)
AKAP82	A-kinase anchoring protein 82 (gene symbol)
AMEL	Amelogenin (gene symbol)
BLAST	basic local alignment search tool
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EGFR	epidermal growth factor receptor
FACS	fluorescence activated cell sorting
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
hGH	human growth hormone
LOS	lack of sharing
miRNA	microRNA
NCBI	national centre for biotechnology information
NMR	nuclear magnetic resonance
nt	nucleotide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PLP	proteolipid peptide (gene symbol)
PSASM	position specific aptamer scoring matrix
PSSM	position specific scoring matrix
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
SDS	sodium dodecyl sulphate
SELEX	systematic evolution of ligands by exponential enrichment
SEM	standard error of mean
siRNA	short interfering ribonucleic acid
SPAM1	sperm adhesion molecule 1
SRY	sex reversal Y (gene symbol)
TB-RBP	testis brain RNA binding protein
UTR	untranslated region
UV	ultraviolet
X-sperm	haploid sperm carrying X-chromosome
Y-sperm	haploid sperm carrying Y-chromosome
ZP3	zona pellucida glycoprotein 3

INTRODUCTION

The sex of an animal is arguably the most economically influential genetic trait, however, current sex-selection methods, although functional, are hindered by high cost. More efficient methods, if developed, could bring sex-selection into mainstream animal production and contribute to a more sustainable industry. By controlling the sex-type of sperm that participate in fertilization, offspring sex-selection can be achieved. As a prerequisite to the development of a new sex-selection strategy, sex-specific markers in sperm need to be identified. All previous efforts to resolve any difference in X- and Y-sperm, other than DNA content, have ultimately failed, and DNA-based selection, using flow-cytometry, is expensive and reduces the fertility and vigour of sex-sorted sperm. This study proposes the development of sex-specific aptamer probes that would exclusively bind to one sex of sperm. Aptamers, a class of molecules used for molecular detection, are short, single stranded, DNA or RNA molecules that adopt stable sequence-dependent secondary structures that in turn bind to specific targets in a lock-and-key conformation.

The goal of this research was to develop aptamer probes to X- and Y-sperm by screening a large randomized library of aptamers against flow-sorted bull sperm using a modified systematic evolution of ligands by exponential enrichment procedure (SELEX). Selected aptamers were characterized by *in vitro* and *in silico* methods to determine if aptamer selection was successful and if selected candidate aptamers were sex-specific.

This research shows that aptamers can be quickly selected to sperm features and can be useful tools similar in application to antibodies. The use of aptamers for identification of sex-specific biomarkers in sperm represents a novel approach to an old problem and studies are ongoing to determine applicability of aptamers for sexing sperm.

CHAPTER 1: REVIEW OF THE LITERATURE

1.1. Are X- and Y-sperm different?

1.1.1. Introduction

Whether X- and Y-sperm actually differ significantly continues to be a source of debate among researchers. Presence of X or Y chromosome and the associated difference in DNA content is one clear difference; however, mature sperm generally are thought to exist as a population of phenotypic clones, with no observable or detectable differences, despite each having a unique genotype. However, significant advancements in our understanding of mammalian sperm function have transformed the once accepted view of the spermatozoa as solely a transporter of the paternal genome. Emerging evidence suggests that spermatozoa are far more metabolically active than originally thought and may display phenotypic molecular differences. This is important for the development of a novel sperm sexing technique as in order to sort X- and Y-sperm there is a need for some detectable qualitative or quantitative differences between the X- and Y-sperm.

1.1.2. Transcript sharing ensures X- and Y-sperm should not differ

During spermatogenesis chromosomal homologues separate and spermatids become genetically unique haploid cells (Braun *et al.*, 1989). Since RNA is transcribed from genomic DNA, allelic differences between homologous chromosomes would lead to different RNA profiles between sperm. However, even though sister spermatids are genetically different they remain mostly phenotypically equivalent due to the exchange of cytoplasmic constituents including RNA transcripts (Braun *et al.*, 1989). Sharing of transcripts, gene products, and/or organelles, compensates for any defective alleles and

creates a population of phenotypic sperm clones each with different genotypes (Braun *et al.*, 1989; Guo and Zheng, 2004). Gametic equivalence is designed to ensure that sperm are evenly matched, and equally capable, of successfully fertilizing the oocyte. Sharing is made possible due to small intercellular bridges 0.1-2 μm in diameter (Guo and Zheng, 2004) connecting developing spermatozoa throughout spermatogenesis, that remain until after nuclear condensation takes place.

Bridges form as a result of incomplete cytokinesis during mitotic and meiotic cell divisions (Braun *et al.*, 1989). An illustrative experiment, proving product sharing between sperm was performed using transgenic mice hemizygous for the human growth hormone (hGH) gene. In this experiment, although the transgene is only transmitted to half the spermatids, 91% (n=2185) of the analyzed sperm contained the hGH protein (Braun *et al.*, 1989). However, hGH is a small soluble protein, therefore this experiment proves sharing with a favorable candidate protein; other less-soluble, or membrane, proteins might be less easily shared.

Transcript sharing of sex-linked genes is imperative as proteins required for functional spermatozoa are present exclusively on both the X- and Y- chromosome (Guo and Zheng, 2004). An example of sex-linked gene sharing is the X-linked AKAP82 gene, which encodes a major fibrous sheath protein that is transcribed post-meiotically, yet is present, and required, in all cells (Turner *et al.*, 1998). It remains to be elucidated if product sharing between sperm is a free-passage type mechanism or an internally (or externally) regulated mechanism.

If transcript sharing is a free-passage type mechanism it is unlikely that deliberate allelic differences could occur between sperm. However, if controlled mechanisms are available to either actively share certain transcripts, or preclude sharing of certain transcripts, allelic differences would be possible. One proposed mediator of sharing is the testis brain-RNA binding protein (TB-RBP), and its orthologous DNA-binding protein Translin, which have been shown to be involved in the intracellular, and intercellular, transport of many RNAs, including some sex linked transcripts, during spermatogenesis (Gu *et al.*, 1998; Hecht, 2000). Similar to the AKAP82 protein, the AKAP4 fibrous sheath scaffolding protein is X-linked, post-meiotically expressed, and essential in all spermatozoa (Morales *et al.*, 2002). The AKAP4 mRNA is bound by TB-RBP in the nucleus and remains associated during transport from the nucleus to the cytoplasm and also as it passes through intercellular bridges between spermatids (Morales *et al.*, 2002). During spermatogenesis transcription and translation become un-coupled, and mRNA is stored for use after the nucleus is shut down (Miller and Ostermeier, 2006). The ribonucleoprotein complex formed between TB-RBP and certain mRNA only dissociates just prior to translation suggesting that TB-RBP may also mediate translational suppression and/or mRNA stability (Morales *et al.*, 2002). TB-RBP-null mice display disrupted spermatogenesis and increased rate of cell apoptosis; however, they are fertile (Hogveen and Sassone-Corsi, 2006).

Precluding transcript, or gene product, sharing could also result in allelic differences between sperm. The lack of sharing (LOS) hypothesis has been shown using Sperm Adhesion Molecule 1 (Spam 1), a sperm membrane protein, which is involved in various

fertilization processes (Cherr *et al.*, 2001). Spam1 RNA is not shared or associated with intercellular bridges due to compartmentalization of the transcript and anchoring to the cytoskeleton (Martin-DeLeon *et al.*, 2005). Sequestering of the transcript followed by the immediate insertion of protein into membrane post-translation results in biochemically different sperm with respect to the Spam1 allele (Zheng *et al.*, 2001). These biochemical differences in Spam1 furthermore can manifest as functional differences, in that sperm made to overexpress Spam1 are less fertile due to retention of cytoplasmic droplet (Martin-DeLeon *et al.*, 2005). RNA-localization in sperm is not surprising as this regulatory phenomenon is well established in oocytes for pattern formation and embryo compartmentalization (Gavis and Lehmann, 1994; Dahm and Macchi, 2007). Functional differences between sperm have also been observed as a result of unequal sharing of the t-complex (although the exact mechanism is not known). Male mice heterozygous for an inversion mutation in the t-complex do not follow typical Mendelian segregation and transmit the mutated t-haplotype to almost all of their offspring despite producing 50% wildtype sperm [for review see: (Hendriksen, 1999; Lyon, 2003)].

If transcript sharing is a controlled process, it is likely mediated to some extent, by conserved elements in the 3'-UTR of RNA. For example, conserved elements in the 3'UTR of testis mRNA have been identified as required for TB-RBP mediated intracellular, and intercellular transport via cytoplasmic bridges (Gu *et al.*, 1998). On the other hand, conserved elements in the 3'-UTR of Spam1 RNA have been shown to be required for compartmentalization of RNA which inhibits cellular transport (Martin-DeLeon *et al.*, 2005). Sharing might be therefore transcript self-regulated based on the

presence or absence of certain signals in the 3'UTR. This mechanism acting through transcriptional variations in 3'UTR, might allow paternal deferred control over the molecular makeup in post-meiotic sperm.

1.1.3. Evidence for differences between X- and Y-sperm.

The total amount of DNA in sperm is the most obvious difference between X- and Y-sperm and serves as the sorting index for commercial sex-sorting by flow cytometry. The Y-chromosome is slightly smaller and therefore Y-sperm contain less total-DNA than X-sperm. DNA content differences have been calculated in at least twenty-three different species ranging from 2.3% in possum to 7.5% in chinchilla (Garner, 2006).

The volume of the sperm head differs proportionally to the DNA content and since X-sperm are carrying a larger 'package' they have been shown to have slightly larger volume (3.5-4% in bovine) (van Munster *et al.*, 1999). No other morphological difference (head length, head width, tail length) has been shown to be statistically significant in X- and Y-sperm or precursor cells (Hossain *et al.*, 2001; Garner and Seidel, 2008). Various 'swim-up' procedures comparing motility and/or density of X- and Y-sperm have not found any significant difference (Han *et al.*, 1993).

Using immunological based approaches to differentiate X- and Y-sperm was initially conceived after Eichwald and Silmsker first described a male specific antigen in 1955 that caused female mice to reject skin transplants derived from syngenic male mice (Eichwald and Silmsker, 1955). However, several reports failed to identify an H-Y antigen on Y-

chromosome sperm (Hendriksen *et al.*, 1993; Howes *et al.*, 1997; Sills *et al.*, 1998). Sex-chromosome specific proteins on the sperm membrane have been reported (Blecher *et al.*, 1999); however, this finding has not been confirmed by others or applied to any sperm sexing approach. An intracellular (cytoplasmic) 40 kDa (pI ~ 5.3) X-linked protein has been detected only in X-sperm via 2D-gel electrophoresis but the identity of this protein has not been determined (Howes *et al.*, 1997a). And high-resolution 2D gel electrophoresis of membrane enriched proteins failed to identify any difference in expression between X- and Y-sperm (Hendriksen *et al.*, 1996).

1.1.4. Sex-selection in nature

Calving records from 642,401 dairy and beef cows in the Irish national 2002 to 2005 database showed that, despite yearly variations, the overall sex ratio did not significantly differ from the expected 50:50 ratio (Berry and Cromie, 2006). However the month of calving, dam parity, and the use of artificial insemination did affect the sex ratio ($P < 0.05$). Also, if the immediately previous calf born is male the odds of another male are increased 1.01-1.07 fold (Berry and Cromie, 2006) or 3.74 fold (Roche *et al.*, 2006). In particular, human intervention into natural animal breeding can affect sex-ratio.

Conflicting results have been reported for sex ratio skewing dependant on AI timing with respect to ovulation (Wehner *et al.*, 1997; Rorie *et al.*, 1999). For IVF, the sex-ratio is shown to be skewed toward males after 1h (64.4% male) and 5h (67.3% male) of sperm-oocyte co-incubation but not 18h (52.3% male) (Iwata *et al.*, 2008). Also, when IVF is performed immediately following first polar body extrusion the sex ratio is found to skew toward females (67% and 58% female at 2-cell and 8-cell stage respectively), while if

delayed 8h the sex ratio skews towards males [69% and 72% male at 2-cell and 8-cell stage respectively (Dominko and First, 1997; Gutierrez-Adan *et al.*, 1999)]. Similar results have been seen *in vivo*: when ewes are inseminated 5 h preceding ovulation, more females are born; while insemination 5 h post-ovulation leads to more males (Gutierrez-Adan *et al.*, 1999). Therefore, it seems likely that under certain circumstances the sex-ratio can deviate from the expected 50% male to 50% female ratio.

Naturally occurring maternal influence over sex of offspring has been reported in various species [for review: (Rosenfeld and Roberts, 2004)] which suggests presence of an endogenous sex-selection mechanism and provides support for potential of a sex-specific marker in sperm. The rationale for maternal control over sex of offspring is derived from the Trivers and Willard hypothesis, which states that as the maternal condition or status declines, it is more evolutionarily beneficial for a female to produce female offspring (Trivers and Willard, 1973). Likewise, under good maternal condition, or maternal dominance, it would be more beneficial for a female to produce more male offspring (Sheldon, 2004). The basis for the Trivers and Willard hypothesis is that in a polygynous population, strong females (maternal dominance) will in turn produce dominant males that will be more likely to breed and pass on genetic information. However, females in poorer condition have a better chance of passing down genetic information through their daughters because their sons will be lower ranking males and not breed as successfully.

Maternal condition has been linked to nutritional and environmental stress factors providing a confirmation for the Trivers and Willard hypothesis in mammals. Mice fed a

low-fat diet produced only 32.2 male per 100 female pups, while the control diet did not deviate from the equal male:female ratio (Rivers and Crawford, 1974). Interestingly, the altered sex-ratio is a result of loss of males *in utero* not an increase in females. Similarly, mice fed a diet low in saturated fat produced a 0.39 male:female ratio, while the opposite effect, 0.67 male:female ratio, is achieved when mice are fed a diet very high in saturated fat (Rosenfeld and Roberts, 2004). Interestingly, only older 20-week-old virgin-mice showed the skewing effect in response to diet but not 10-week-old mice. This indicates that maternal age might be important in sex-ratio skewing possibly due to different response to a perceived environmental stress due to diet. A similar decrease in males has also been shown in Chinese hamsters following caffeine treatment prior to mating (Weathersbee *et al.*, 1975). Environmental conditions might also affect sex ratio. A 1°C increase in average maximum daily temperature during the week prior to conception is correlated with a 1% increase in the likelihood of male calf in dairy cattle (Roche *et al.*, 2006). Paternal influence has also been linked to altered sex-ratio where chemical exposure of men has been shown to decrease the male:female ratio significantly (Milham, 1993; Mocarelli *et al.*, 2000), however the mode of action is not known.

Even if the Trivers and Willard hypothesis is confirmed in mammals, it has been commonly assumed that any sex ratio variation is due to sex selective embryo loss (Boklage, 2005). Yet, such mechanism seems extremely wasteful and expensive from evolutionary point of view, and some studies do suggest that females might be able to select offspring sex preconception (Kruger *et al.*, 2005; James, 2006). High levels of testosterone in the mammalian follicle are associated with a subsequent male embryo.

This is potentially caused by testosterone modifying the developing oocyte in such a way that it is more likely to be fertilized by a Y-sperm (Grant *et al.*, 2008). The authors suggest that perhaps the increased level of testosterone alters the natural expression of zona pellucida proteins affecting the sperm-oocyte interaction, or that interaction between cumulus cells and sperm are altered in some way to either exclude, or permit, one sex of sperm (Grant *et al.*, 2008). Female testosterone levels are known to rise as a response to chronic stress (Powell *et al.*, 2002; Yoon *et al.*, 2005) but it is unknown if this rise in testosterone also occurs in the follicle. This mechanism might explain the skewed sex ratio in humans during periods of war (Macmahon and Pugh, 1954) as a response to behavioral stress mediated through a testosterone pathway in the female. The level of follicular testosterone is likely influenced by a combination of genetic and environmental factors potentially resulting in a different predisposition to one sex or the other during each cycle (Grant and Irwin, 2005). A preconception embryo predisposition to one sex of sperm, particularly if the bias is toward Y-sperm fertilization, might explain the low fertility found when sex-sorted sperm (normally X-sorted) is used (Grant and Chamley, 2007).

1.2. Molecular activity in sperm

In order for a molecular difference between X- and Y-sperm to occur, mechanisms to overcome transcript sharing, nuclear condensation (no transcription), and loss of ribosomes (no translation), would need to exist. In light of recent progress in sperm research, the sperm can no longer be viewed as a cell whose only function is to transport

the male genome. Evidently, mammalian sperm are more active than originally thought - particularly with respect to functional RNA mechanisms.

1.2.1. The sperm transcriptome

The debate over whether sperm-RNA exists, or plays any role in sperm function, stemmed from the fact that during spermatogenesis, the nucleus is progressively shut down and spermatids shed their cytoplasm resulting in the loss of cytoplasmic constituents including ribosomes (rRNA) and, theoretically, mRNA (as the cytoplasm is the main storage area for mRNA). However, there appears to be some degree of organized retention of certain RNA molecules. Most interestingly, all of the cytoplasmic ribosomal RNA is shed while a sub-population of mRNA and regulatory RNA are retained (Miller *et al.*, 1999). This bias in RNA retention suggests the presence of a yet-to-be-defined mechanism for selective RNA retention, most likely via stabilizing proteins or protective vesicles. RNA in sperm has been shown to be located in the nucleus, cytoplasm, mid-piece, and mitochondria (Miller and Ostermeier, 2006). Recently, modern molecular 'omic approaches have been applied to fully annotate the sperm transcriptome identifying ~3000 unique RNA molecules. Transcripts encoding transcription factors are particularly well represented in the sperm transcriptome as well as transcripts identified as important in cell signaling and embryonic development. If a mechanism is available for postmeiotic transcription/translation of Y-linked genes such as *Sry* (Capel *et al.*, 1993) or *Zfy1/2* (Nagamine *et al.*, 1990) and X-linked genes such as *SPANX* (Westbrook *et al.*, 2000) or *Ubelx* (Hendriksen *et al.*, 1995), it could result in sex-unique transcripts, proteins, and phenotypes.

1.2.2. Transcription in sperm

In somatic cells, RNA is synthesized in the cell nucleus by RNA-polymerase enzymes; however, during spermatogenesis the sperm genomic DNA becomes highly condensed and is thought to be incapable of transcription. Prior to DNA condensation mRNA transcription activity is high in the haploid cell, however, at this point spermatids are still attached by intercellular bridges (Dadoune *et al.*, 2004). Affymetrix microarray comparison of transcriptome differences between spermatogonia (mitotic) and spermatocytes (meiotic) in mice shows that differential gene expression does occur during different stages of spermatogenesis and that there is an increase of transcription activity in spermatocytes potentially to build-up transcripts for use after nuclear condensation (Rossi *et al.*, 2004). Transcription during spermatogenesis is expected, however, transcription in mature sperm is still debatable.

RNA polymerase, acting on endogenous transcripts, has been identified in the head and tail fractions of ejaculated bovine spermatozoa, although, it could be argued that these studies could be generating misleading results due to potential for somatic cell contamination in their procedure(s) (Fuster *et al.*, 1977; Hecht and Williams, 1978). In contrast to these early studies, Grunewald and colleagues found that when radio-labeled uridine triphosphates are used for *in vitro* labeling experiments, no radioactive RNA is found in the sperm; apparently confirming that mature sperm are indeed transcriptionally silent (Grunewald *et al.*, 2005). Miller and Ostermeier disagree with Grunewald's interpretation stating: "incubations were carried out under conditions that would not have favoured the up-regulation of a dormant transcriptional capacity" reiterating at least the

potential for transcription in mature sperm (Miller and Ostermeier, 2006). Interestingly, in all the large-scale genomic characterization studies performed to date, transcription factors are the most abundant transcripts present in mature sperm (Ostermeier *et al.*, 2002; Dadoune *et al.*, 2005; Zhao *et al.*, 2006). This would seem to suggest that sufficient regulation mechanisms are present in sperm for transcription to take place (assuming these transcripts can be translated).

Protamine-mediated condensation of genomic DNA is thought to be responsible for transcriptional silencing, however, recently it has been shown that there is non-random distribution of histone-enriched regions throughout the mature sperm genome. Histones package DNA more loosely compared to protamines, allowing access to the DNA by the transcriptional apparatus. When a cDNA library is constructed containing the non-protamine regions, clusters of potential binding sites for transcription factors are identified (Pittoggi *et al.*, 2001). It is further shown that for all of the binding sites identified the corresponding transcription factor protein complexes are all endogenously present in mature sperm heads (Pittoggi *et al.*, 2001).

While traditional transcription in sperm is still debatable, a reverse-transcriptase mechanism that is endogenous to sperm has been identified (Miller, 2000; Sciamanna *et al.*, 2003). When sperm are incubated with foreign β -galactosidase RNA, the RNA is taken up, and reverse-transcribed into cDNA. Detection of the β -gal cDNA by PCR is abolished with DNase I digestion, but is not affected by RNase treatment, confirming that reverse transcription occurred (Sciamanna *et al.*, 2003). When these sperm are used for

insemination the founder animals displayed a mosaic pattern of β -gal expression suggesting that the cDNA is not incorporated into the genome. The same group later showed that when exogenous DNA is incubated with sperm it is readily taken up and transcribed into RNA, the RNA is then spliced, and then reverse transcribed into cDNA (Pittoggi *et al.*, 2006). The authors suggest that transcription, splicing, and reverse transcription mechanisms, are typically dormant in sperm and are naturally activated at fertilization or after interaction with foreign nucleic acids (Pittoggi *et al.*, 2006). These mechanisms allow for the formation of non-integrated biologically active retro-genes, however, the natural function has not yet been recognized and these results remain to be independently confirmed.

1.2.3. Non-coding RNA:

A new role of RNA is emerging that is independent of the *central-dogma* theory of RNA as a mediator between DNA and functional proteins (for review of RNAi see (Caplen and Mousses, 2003; Morris *et al.*, 2004; Engels and Hutvagner, 2006). Small non-coding RNAs acting as regulators of gene expression have been confirmed during mitosis, meiosis, and haploid round-cell stages of spermatogenesis (Shoji *et al.*, 2005). When endogenous genes are targeted in spermatocytes using siRNAs the resulting phenotypes are analogous to control null-mutant mice indicating effective gene silencing by RNAi (Shoji *et al.*, 2005) Antisense-microarray identified 68 novel siRNAs in sperm from six normal human sperm donors (Ostermeier *et al.*, 2005). A second microarray study using known siRNA probes found 191 miRNAs in seven human donors in which 24 were confirmed by real-time PCR (Amanai *et al.*, 2006). Consistent with the presence of

siRNAs, all the members of the RNAi machinery are also present in haploid sperm cells including Dicer, RISC, and members of Argonaute protein family, however RNAi activity has not yet been confirmed (Kotaja *et al.*, 2006a; Kotaja *et al.*, 2006b).

1.2.4. Translation in sperm

The intuitive reason for the presence of RNA in sperm is that it serves as template for translation at some point during the life of the sperm. The generally accepted view of spermatozoa is that they are completely translationally silent due to the fact that they shed their cytoplasmic ribosomes during the late stages of spermatogenesis. However, labeled amino acids are incorporated into polypeptides during sperm capacitation in human, mouse, bovine, and rat sperm (Gur and Breitbart, 2006). The only ribosomes present in mature spermatozoa are mitochondrial 55S ribosomes. The incorporation is abolished by the mitochondrial 55S ribosomal inhibitors (D-chloramphenicol, tetracycline, gentamycin), but is not affected by the cytoplasmic 80S ribosomal inhibitor (cycloheximide), indicating translation in sperm stems from the mitochondria (Gur and Breitbart, 2006). The proposed scenario is that nuclear-encoded transcripts stored in the sperm are translated as needed by the mitochondrial ribosomes (Gur and Breitbart, 2006). This study found translation of 22 proteins including: protein kinase C (PKC), epidermal growth factor receptor (EGFR), progesterone receptor (PR), Na-K-ATPase, and the catalytic subunit of protein kinase A. One of the affected proteins AKAP110 is interesting as AKAP4 and AKAP82 have been shown to be sex-linked, post-meiotically expressed, and under organized sharing control by TB-RBP (Turner *et al.*, 1998; Morales *et al.*, 2002). Inhibition of protein translation resulted in decreased sperm motility,

capacitation, and fertilization rate (Gur and Breitbart, 2006). The authors hypothesize that protein synthesis in spermatozoa serves either to replace proteins that have been degraded or produce capacitation specific proteins (Gur and Breitbart, 2006). However, this mechanism could also be used to make up for unevenly shared proteins or to generate sex-specific differences between X- and Y-sperm.

1.3. Sperm sorting by flow cytometry

1.3.1. Sorted semen

The justification for producing sexed semen depends on the species of intended use. In livestock, the sex of an animal is often the most important genetic trait with significant associated financial implications (Seidel, 2003a; Johnson *et al.*, 2005). In an industry dominated by cost-efficiency, sorting would allow for superior herd management, selection of sex-limited traits, and faster progress of genetic lineage (Johnson *et al.*, 2005).

In humans, sexing would minimize the occurrence of X-linked genetic diseases; many of which are extremely debilitating in man (Seidel and Johnson, 1999). Theoretically, half the sons born to carrier females will display the affected trait while daughters will not. Therefore sex-sorting would increase the likelihood of bearing a healthy female child. The first successful human clinical trial of sperm sexing for prevention of an X-linked condition was achieved in 1995 (Levinson *et al.*, 1995) and today has become mainstream medicine (MicroSort[®]; Fairfax, Virginia).

1.3.2. History and Methods

As early as 1925, researchers at the Wisconsin Agricultural Experiment Station experimented with centrifugation methods to separate X- and Y-sperm – already acknowledging the benefit(s) of controlling the sex of offspring in livestock (Lush, 1925). Investigations continued over the 20th century to identify some quantitative or qualitative difference in X- and Y-sperm. Currently the only proven, and commercially available, method for sorting sperm is flow cytometry based on differential DNA content between X- and Y-sperm. An estimated 30,000 normal appearing offspring, primarily cattle, were produced worldwide between 2000 and 2005 (Johnson *et al.*, 2005), using the Beltsville Sperm Sexing Technology developed at the US Department of Agriculture in Beltsville, Maryland. The Beltsville sexing technology uses the fluorescent Hoechst 33342 DNA binding stain to resolve X- and Y-sperm based on DNA content and the membrane impermeable dye propidium iodide (PI) that quenches the Hoechst stain in dead cells so they can be gated from the stream. After staining of DNA the sperm are passed through the flow cytometer and illuminated using an argon laser (351nm, 150mW) (Spinaci *et al.*, 2005). Since X-sperm contain more DNA they bind more of the Hoechst stain compared to Y-sperm and therefore fluoresce more intensely.

The use of the flow cytometer to resolve sperm required significant development prior to widespread usage of the technology. Initial attempts to measure DNA content failed due to the uniquely small, non-spherical, flat shape of the sperm head resulting in problems with detection (Johnson, 1997). Dean *et al.* found that by using specially shaped sample injection tubes to hydrodynamically orientate the sperm with respect to the laser, much

better results could be obtained (Dean *et al.*, 1978). In addition to a special nozzle, flow-cytometers used for sperm sorting also require a forward fluorescence detector, in place of the side scatter detector, to be able to measure fluorescence from both the flat side (0°) and edge (90°) of sperm (Johnson *et al.*, 2005). There are three high-speed sorters capable of being adapted for sperm sorting: the FACS Vantage (Becton-Dickinson Inc.), the Altra (Beckman-Coulter Inc.), and the MoFlo (Dako-Cytomation Inc.); the latter of which is used commercially (Johnson *et al.*, 2005).

1.3.3. Efficiency of sorting by flow cytometer

Using the MoFlo (Dako-Cytomation Inc.) sperm can be sorted at a rate of 3000-4000 live sperm per second for cattle (Garner, 2001). This is an impressive achievement, however, when evaluated in the context of the number of sperm required for insemination (2 million live sperm / dose) the sorting speed remains a limiting factor of the technology. The purity of sorted samples as determined by quantitative real-time-PCR of sex specific regions ranges from 89%-95% for X-sperm and 86-96% for Y sperm (Garner, 2001; Garner, 2006; Parati *et al.*, 2006). The ability of a flow cytometer to distinguish between the sperm, referred to as the sorting index, is calculated by multiplying the head profile area (μm^2) by the percent DNA difference. The higher the index score the better the flow-cytometer can resolve differences between sperm. For example, in boars the head area is $37.5\mu\text{m}^2$ and the DNA content difference is 3.6% resulting in a high sorting index score of 115 (Garner, 2006). By comparison, horses and humans have much smaller sperm resulting in lower index scores of 59 and 31 respectively (Garner, 2006).

1.3.4. Damage to sperm

The entire process of flow sorting subjects each spermatozoa to chemical stresses, temperature fluctuation, high dilution, high pressure, UV radiation, and significant mechanical forces (Spinaci *et al.*, 2005). Flow sorting has been observed to alter the sperm membrane causing premature capacitation resulting in shorter lifespan of sperm *in vitro* (Maxwell *et al.*, 1998). Damage to sperm translates to a 42.2% and 45.7% decrease in pregnancy rate in sows for low and high dose deep intrauterine inseminations respectively in flow-sorted samples as compared to controls (Vazquez *et al.*, 2003). Similarly, non-surgical insemination doses of 50 million sperm resulted in a 33.3% pregnancy rate in sows using sex-sorted sperm compared to 54.5% using un-sexed control sperm (Grossfeld *et al.*, 2005). A standard dose of sexed sperm in Holstein-Friesian cows resulted in a 26% decrease in pregnancy rate compared to controls (Andersson *et al.*, 2006). Cryopreservation of sperm appears to amplify damage caused by the flow sorting, as flow-sorted sperm that are subsequently cryopreserved and thawed undergo the acrosome reaction more rapidly as compared to un-sorted control samples (Moce *et al.*, 2006). The effect of flow sorting may not be limited to decreased fertility, as abnormalities during gestation have also been reported using sex-sorted sperm. Various problems with embryonic development have been reported in sows where an average decrease in farrowing rate of 51% is obtained with insemination of sex-sorted sperm. Also 15% of sows aborted fetuses 20-50 days after insemination compared to only 1% in control groups indicating that early developmental abnormalities are prevalent (Vazquez *et al.*, 2003). The sperm chromatin structure assay found that even after the gating of dead sperm during the sorting process the sorted sample still showed higher

chromatin damage (Boe-Hansen *et al.*, 2005). The cause of DNA damage is not the Hoechst stain as exposure to the dye alone does not significantly alter pregnancy rate, farrowing rate, and/or litter size (Parrilla *et al.*, 2004). Also, there is no mutagenic effect in flow-sorted boar sperm due to combination of staining and UV-laser (Parrilla *et al.*, 2004).

1.3.5. Practicality of flow-sorting

Twenty years after the initial investigations of sorting sperm by flow cytometry there is little doubt as to the effectiveness of this technology. Although the technology is viable in many livestock animals [cattle (Bodmer *et al.*, 2005; Andersson *et al.*, 2006), swine (Grossfeld *et al.*, 2005), sheep (Hollinshead *et al.*, 2002), equine (Lindsey *et al.*, 2001)], it hasn't yet achieved widespread use in animal production. Only the dairy industry has embraced sperm sorting, as there is a clear benefit to producing female animals, which justifies the added cost premium. Sexed sperm can be especially valuable in situations where there is shortage of a particular breed due to import restrictions, popularity, or when quick herd expansion is required. Sexing is becoming particularly important with current tendencies for development of specialized terminal and maternal crossbred lines and separate sex feeding programs. Other applications will surely arise when reasonably priced sexed sperm become widely available for animal industry. The 'practicality' of using sexed sperm for insemination becomes a joint function of the speed, fertility, processing time, and ultimately economics.

Sperm sorting by flow-cytometry remains inaccessible to the average end-user due to the premium of \$30-\$50 per dose over regular unsexed sperm depending on quality of bull, purity, shipping distances, etc (Seidel, 2003a). Justifying this added cost for an average-scale farmer can be difficult given the decreased fertility of sperm and the less than 100% purity in sample. The effect of less than perfect purity was exemplified in a recent field trial where only 82% of animals were born of the desired sex after insemination with sexed sperm (Andersson *et al.*, 2006). Experts suggest that premiums in the range of \$10 per dose would likely be required to make sexed sperm economical for most cattle populations (Seidel, 2003a).

Currently, flow sorting is unrealistic for sperm producers as well given the inordinate cost of establishing a sorting operation. The purchasing cost of a suitable flow cytometer with sorting options can exceed US\$ 300,000 (Seidel, 2003b) and requires specialized water, stable electricity, and temperature/humidity control typically only found in sophisticated laboratories (Seidel, 2003a). The maintenance contracts for equipment and training of personnel quickly push the cost of sperm sorting beyond the reach of typical sperm producers. G.E. Seidel, a lead researcher in the field, has estimated the cost of establishing a small sorting program including licensing fees, equipment, and personnel, to be approximately US\$ 2 million. As a result sperm sorting remains a lab-based technique that doesn't transfer well to a field based sorting program.

1.3.6. Future of sperm sorting by flow-cytometry

Sorting sperm by flow cytometry is still in its infancy and will continue to be developed in the near future. Current research in the field is focused on decreasing the mechanical

stress on the sperm as this has been identified as the primary contributor to the observed decrease in fertility (Suh *et al.*, 2005). Development of dedicated flow-sorters exclusively for sperm sorting without all the ‘bells and whistles’ of current systems should significantly lower equipment costs. The widespread commercialization of sexed sperm, particularly in agriculture, is dependent on several parallel technologies. Firstly, given the added premium of sexed sperm, low-dose insemination strategies will be influential in making sperm sexing economical (Vazquez *et al.*, 2005). A current limiting factor to the expansion of sexed sperm has been the need for better semen storage techniques (Johnson *et al.*, 2000). Advances in cryopreservation of semen are expected and will allow for the commercialization of the technology into species other than cattle.

1.3.7. Alternatives to sorting by flow-cytometry

Currently there is no alternative to flow sorting for the sexing of live mammalian sperm. All previous attempts at alternative methods have resulted in failure. The number of sperm analyzed per unit time with a flow-cytometer is limited because each sperm must be measured independently. Alternatively, if a sex-specific marker could be identified, sperm could be sorted in parallel allowing millions of sperm to be sorted in seconds (not hours). This type of selection would also allow farm based sorting and would eliminate issues with accessibility, long-term storage of semen, and likely negate the need for low dose insemination.

1.4. Research objectives

Currently, no sex-specific marker has been identified on sperm. With the commercial availability of high purity flow-cytometry sex-sorted sperm there is now potential to develop sex-specific aptamer probes to resolve slight molecular differences between X- and Y-sperm. Identification of sex-specific markers on sperm would allow the improvement of existing sex-sorting methods, or the development of new sex-sorting technologies. To this end, three specific objectives were set:

- Develop method for selection of aptamers to live sperm features.
- Select aptamers to flow-cytometry sex-sorted X-sperm and Y-sperm.
- Determine if selected aptamer(s) are sex-specific.

CHAPTER 2: SELECTION OF SEX-SPECIFIC APTAMER PROBES TO SPERM.

2.1. Introduction

The role of nucleic acids in biology goes far beyond the simple storage of genetic information. Emerging roles such as RNA interference, micro RNA, riboswitches and ribozymes, are establishing nucleic acids as part of the nanotechnology arsenal, which allow manipulation of biological systems at microscopic level. Short single stranded nucleic acids, or oligonucleotides, will fold onto themselves forming elaborate, sequence dependent, three-dimensional structures. Ellington and Szostak first described an RNA sequence that would fold in such a way to create a specific binding site for small ligands. These nucleic acid molecules are termed aptamers from the Latin word 'aptus' meaning 'to fit' (Ellington and Szostak, 1990). At the same time, a separate laboratory isolated high-affinity nucleic acid ligands to T4 DNA polymerase from a random library using a systematic evolution of ligands by exponential enrichment procedure (SELEX) (Tuerk and Gold, 1990).

Aptamers are oligonucleotides that have folded in such a way that they can bind specific targets in a lock-and-key type conformation usually associated with proteins. Aptamer structure is dependent on the oligonucleotide sequence not only forming two dimensional stems, loops, bulges, hairpins, but also more complex three dimensional structures such as pseudoknots, triplexes, or quadruplexes (Stoltenburg *et al.*, 2007). Aptamers have been

developed to a myriad of targets including inorganic molecules, organic molecules, nucleotides, cofactors, amino acids, carbohydrates, antibiotics, various proteins, and entire cells and organisms (Stoltenburg *et al.*, 2007). The ability of aptamers to bind a specific target, even in the presence of a complex mixture, has made them valuable tools for molecular recognition, biosensor application and affinity purification.

Aptamers in many ways rival antibodies as a tool for molecular detection. Aptamers are stable long-term, infinitely amplifiable, can be modified with various tags or backbones, and are cheap to synthesize once the sequence is developed. Also, since aptamers are developed purely *in vitro*, no animals are needed; and the immunogenicity is usually low even when administered at high dosage (Nimjee *et al.*, 2005). Aptamers are known for high specificity, being able to discriminate slight differences, such as a single methyl group, ten times better than the corresponding antibody (Jenison *et al.*, 1994; Jayasena, 1999). Perhaps the greatest feature of aptamers is that no *a priori* knowledge is required about the nature of the target for selection.

Here we describe the development of SELEX approach to evolve sex-specific aptamers to differentiate X- and Y-chromosome bearing bull sperm. Many previous attempts, including antibody and 2D gel approaches, have failed to identify a sex-specific feature in sperm and it is commonly accepted that no such differences exist on sperm surface. Aptamers are an ideal tool in this scenario given their high specificity and wide scope of potential molecule types for sex-specific targets (Nimjee *et al.*, 2005). Also the big

advantage of SELEX is that it can be applied on a presumed difference between X- and Y-sperm without any specific target in mind.

Aptamers are developed by strategically evolving a large random aptamer library through repetitive rounds of selection and amplification for aptamers with desirable characteristics and against aptamers with undesirable characteristics. In the most basic application of SELEX, purified target molecules, such as a protein of interest, are used for selection. Since the original description of the SELEX procedure many variations have been described to adapt the technique (for review: Stoltenburg *et al.*, 2007).

Shangguan *et al.* have described a particular SELEX variation wherein aptamers are selected to entire cells as opposed to purified target molecules (Daniels *et al.*, 2003; Shangguan *et al.*, 2006). Aptamers have been developed by this whole-cell SELEX approach to discriminate cancerous cells from non-cancerous cells (Shangguan *et al.*, 2006). Conceptually, this approach to discriminate two cell types is analogous to developing aptamers to discriminate X-sperm from Y-sperm. To our knowledge, prior to this study, aptamers have not been used in sperm research. The development of a sperm SELEX procedure required adaptation of the existing methods to suit conditions required for non-adherent motile sperm. X- and Y-sperm sex-sorted by flow-cytometry are used here as independent target cell types for the development of sex-specific aptamers.

The sperm-SELEX procedure merged several key techniques to develop aptamers that could discriminate X/Y-sperm and overcome the non-specific DNA-binding known to exist in sperm. Negative selection, or subtractive-SELEX, is a technique used to develop

aptamers capable of discriminating two closely related targets (Geiger *et al.*, 1996).

Negative selection incorporates additional selection steps to closely related target molecules to filter the library of aptamers that bind these related targets. In the sperm-SELEX procedure two rounds of negative selection are performed during each cycle using the opposite sex of sperm to filter the aptamer pool prior to positive selection to the target sex of sperm. Competitor DNA is a technique that minimizes the selection of non-specific aptamers, which bind randomly. Large molar excess, peaking at 1000-fold excess in later rounds, of non-amplifiable competitor DNA occupies non-specific sites, such as positively charged proteins, forcing aptamers to bind to specific targets.

The goal of this research was the development of a SELEX method suitable for selection of aptamers to live-sperm features and subsequently the application of this method for the development of aptamer probes to bull sperm.

2.2. Materials and Methods

2.2.1. Sperm preparation

Unsorted, non-frozen, sperm (Gencor, Guelph; 4 Holstein bulls; 1 bull per selection round) and cryopreserved X- and Y-sperm that were sex-sorted when fresh by flow cytometry (n =5; Holstein bulls), were used as target cells for selection and for aptamer testing. At minimum, sex-sorted samples from three different bulls were used during each selection round (rounds 1-10) to minimize bull-effect (later rounds used sperm from a single bull). Purity of sex-sorted sperm was determined by flow-resort analysis at the time of sorting. Sex-sorted samples used in selection rounds 1-10 were estimated at 90% X-sperm for the X-sperm sorted samples, and 80-85% Y-sperm for the Y-sperm sorted samples. Sperm used in later selection rounds was determined to be 92% X-sperm for the X-sperm sorted samples, and 95% Y-sperm for the Y-sperm sorted samples.

Cryopreserved sperm were thawed at 37°C for 5 min then allowed to cool to room temperature prior to use. Cryopreserved sperm were washed from cryoprotectant medium by centrifugation (400 x g, 7 min, room temperature, Thermo Scientific Microlite centrifuge), and resuspended in selection buffer [1 x PBS (HyClone, Logan, UT, USA) supplemented with 5 mM glucose]. Fresh unsorted sperm was collected and stored at 37 °C until use (60-180 min from collection to processing). Prior to use sperm was allowed to cool to room temperature and was washed as described for frozen sperm to remove seminal plasma.

2.2.2. Aptamer library preparation

A 90-nucleotide DNA aptamer library containing a 36-nucleotide randomized region (theoretical complexity of 4.7×10^{21} aptamer molecules) flanked by fixed primer regions was used for aptamer selection (5'- TCCATCTCTTCTGTATGTCGAGATCTA-(N)₃₆-TAGATCTCCTAACCGACTCCGTTAATT-3') (Roulet *et al.*, 2002) (Figure 1) (Sigma, St. Louis, MO, USA). Asymmetric PCR amplification was used to generate excess of fluorescently labeled single stranded aptamer molecules (Gyllenstein and Erlich, 1988; Sanchez *et al.*, 2004). Amplification was conducted across 4 reactions to avoid inadvertently limiting the aptamer pool complexity during PCR. Amplification was carried out in 50 μ L reactions containing 5 μ L of 1x ThermoPol reaction buffer (New England BioLabs, Ipswich, MO, USA), 2.5 units of *Taq* polymerase, 4 μ M of forward FITC-labeled primer (5'-[FITC]- TCCATCTCTTCTGTATGTCGAGATCTA), 0.1 μ M of reverse primer (5'- AATTAACGGAGTCGGTTAGGAGATCTA-3'), 200 μ M of dNTP mix. Amplification reaction was performed using a Biometra TGradient thermocycler with one cycle of 95 °C for 4 min, 40 cycles of 95 °C for 30 s, 61.6 °C for 30 s, and 72 °C for 15 s, and a final extension step of 72 °C for 5 min. Asymmetric PCR reactions were purified to remove unincorporated FITC-primers using Montage purification column (Millipore, Billerica, Massachusetts). For aptamer purification, asymmetric amplification reactions were pooled and volume adjusted to 400 μ L with TE buffer (10 mM Tris-HCl, pH 8.5, 1 mM EDTA). Aptamer solution was applied to Montage column and filtered by centrifugation (1200 x g, 10 min at 4 °C). After centrifugation, aptamers were eluted from inverted column by incubation with 40 μ L of TE buffer for 1 min followed by centrifugation (1200 x g, 2 min, 4 °C). To monitor

aptamer recovery, and amplification, aptamers were separated by agarose gel electrophoresis [3% agarose, 135V, 30 min, chilled 0.5 x TAE running buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3)]. Fluorescent aptamer concentration was determined by FITC-standard curve produced with FITC-labeled primer using Typhoon Trio system with ImageQuant TL v2005 software (Amersham Biosciences, Piscataway, NJ, USA).

Figure 1. Design of aptamer library used in this study. Aptamer library consists of a 36-nucleotide random region (grey) flanked by two 27-nucleotide fixed primer regions (black). Aptamers were labeled at the 5' end with a single fluorescein isothiocyanate (FITC) molecule. (5'- FITC-TCCATCTCTTCTGTATGTCGAGATCTA-(N)₃₆-TAGATCTCCTAACCGACTCCGTTAATT-3'). Figure adapted from: (Marathias *et al.*, 1996; Chen *et al.*, 2003)

2.2.3. Aptamer selection

Sex-specific aptamers were selected using a modified systematic evolution of ligands by exponential enrichment procedure (SELEX) adapted for use with sperm cells (Figure 2). For aptamer selection to sex-sorted sperm, 200 pmol of aptamer library (1.2×10^{14} theoretical complexity) was used in first round. In total, 20 rounds of aptamer selection were performed with increased selection pressure via negative selection, competitor DNA, decreased aptamer dosage, and increased wash stringency, in later rounds (table 1). Competitor DNA used in this study was derived from salmon sperm genomic DNA sonicated to produce single stranded molecules ranging in size from 587-831 nucleotides (Sigma, St. Louis, MO, USA). Briefly, a typical selection round was performed by first thawing on ice total recovered aptamer solution from previous selection round (~50 μ L total volume). Asymmetric aptamer amplification was carried out across 4 x 50 μ L reactions using 8 μ L of recovered aptamer solution as template in each PCR reaction. Aptamers were amplified and purified by column as described. Agarose gel electrophoresis of a non-amplified/non-purified aliquot (5 μ L of recovered aptamer solution) and an amplified/purified sample was used to determine aptamer concentration before and after asymmetric PCR amplification to monitor FITC label incorporation. Additionally, by comparing the quantity of (unamplified) aptamers remaining after a selection round to the amount used in previous round, the percent aptamer exclusion per round was monitored. Sperm used for selection were prepared as described and held at room temperature in selection buffer during selection procedure. Entire selection procedure was performed at room temperature. Aptamer selection was conducted by incubating aptamers (quantities as per table 1) with 500 μ L of negative target sperm cells

(4×10^5 sperm in selection buffer) for 30 min at room temperature in a light-shielded polypropylene microcentrifuge tube with occasional gentle inversion to mix incubation solution. X-aptamers were negatively selected to Y-sorted sperm; Y-aptamers were negatively selected to X-sorted sperm (Figure 2). After incubation, negative cells were removed by centrifugation (5000 x g, 3 min, room temperature). The supernatant, containing aptamers that did not bind negative cells, was recovered by aspiration (450 μ L) and transferred to 500 μ L (4×10^5 sperm in selection buffer) of negative target cells in a light-shielded polypropylene microcentrifuge tube for an additional negative selection incubation as per above. After the second negative selection, aptamers that did not bind negative cells were recovered as before by centrifugation and aspiration (900 μ L) and transferred to 500 μ L (4×10^5 sperm in selection buffer) of positive target cells in a light-shielded polypropylene microcentrifuge tube. Competitor DNA (quantities as per table 1) was added, and positive selection incubation was performed for 15 min at room temperature with occasional gentle inversion to mix incubation solution. X-aptamers were positively selected to X-sorted sperm; Y-aptamers were positively selected to Y-sorted sperm. Positive target cells were washed once to remove loosely bound aptamers by centrifugation (400 x g, 7 min, room temperature) and resuspending sperm in 1000 μ L of selection buffer and holding at room temperature for 5 min. Candidate aptamers were co-recovered with positive target cells by centrifugation (5000 x g, 3 min, room temperature). Aptamers were released from the positive cell pellet using a two-step sperm lysis procedure (Li *et al.*, 1988; Cui *et al.*, 1989). To the sperm pellet 20 μ L of lysis buffer 1 [20 mM dithiothreitol (DTT), 1.7 μ M sodium dodecyl sulphate (SDS), 1 mg/mL proteinase K, in 1X ThermoPol reaction buffer] was added, mixed gently by

aspiration, and incubated at 56 °C for 60 min, followed by 95 °C for 10 min to inactivate protease. Subsequently, 8 μ L of lysis buffer 2 [50 mM dithiothreitol (DTT), 200 mM KOH] was added and incubated at 65 °C for 10 min. Lysis solution containing the candidate aptamers was neutralized with 8 μ L of neutralizing buffer [300 mM KCl, 900 mM Tris-HCl (pH 8.3)] and frozen at -20 °C until next selection round. Total volume of recovered aptamers after pellet lysis was ~50 μ L.

Figure 2. Selection scheme of sperm-SELEX procedure. Sorted Y-sperm (blue) provide negative selection, and sorted X-sperm (pink) provide positive selection. Figure adapted from: (Marathias *et al.*, 1996; Chen *et al.*, 2003; Shangguan *et al.*, 2006)

Table 1. Conditions of aptamer selection over 20 selection rounds showing type of sperm (sorted, unsorted), amount of aptamer and competitor used, and the selection scheme for each round.

Selection round	sperm used	aptamers used (pmol)	competitor used (pmol)	pos/neg selection (order)
1	unsorted	200	0	pos
2	unsorted	200	0	pos
3	sex sorted	500	0	neg->pos
4	sex sorted	225	0	neg->pos
5	sex sorted	150	0	pos->neg->pos
6	sex sorted	75	0	neg->neg->pos
7	sex sorted	75	0	neg->neg->pos
8	sex sorted	10	1000	neg->neg->pos
9	sex sorted	1	1000	neg->neg->pos
10	unsorted	5	1000	pos
11	sex sorted	1	1000	neg->neg->pos
12	sex sorted	1	1000	neg->neg->pos
13	sex sorted	1	1000	neg->neg->pos
14	sex sorted	1	1000	neg->neg->pos
15	sex sorted	1	1000	neg->neg->pos
16	sex sorted	1	1000	neg->neg->pos
17	sex sorted	1	1000	neg->neg->pos
18	sex sorted	1	1000	neg->neg->pos
19	sex sorted	1	1000	neg->neg->pos
20	sex sorted (neg), unsorted (pos)	1	1000	neg->neg->pos

2.2.4. Aptamer evaluations

Aptamer evaluations by flow-cytometry, fluorescent binding assay, and fluorescence microscopy were applied during selection (rounds 7-11) to assess success of aptamer selection, evolving aptamer sex-specificity, and aptamer binding characteristics. All aptamer evaluations were performed independently from aptamer selection procedure.

For aptamer evaluations, pseudo-selection rounds were performed using pooled sorted X- and Y-sperm (pooled from 4 Holstein bulls after thawing) and unsorted fresh-sperm (1 Holstein bull) prepared as described above for aptamer selection. Selected X- and Y-aptamers (post selection rounds) were re-amplified from aptamer pool used during selection and were purified by column and concentration determined as described above. The initial aptamer library, used here as a non-selected control was amplified by asymmetric PCR (1 pmol as template) using described asymmetric PCR methods to incorporate FITC-label for detection. Competitor DNA was not used during evaluations. To measure degree and specificity of selected aptamers to X- and Y-sperm positive incubations (X-aptamers X-sperm, Y-aptamers Y-sperm) and negative incubations (Y-aptamers X-sperm, X-aptamers Y-sperm) and control incubations (initial-library X-sperm, initial-library Y-sperm) were performed using prepared aptamers (5 pmol in 10 μ L of 1 x ThermoPol PCR buffer) and prepared sorted sperm (4 x 10⁵ X- or Y-sperm in 250 μ L of selection buffer). To measure aptamer binding to unsorted fresh sperm, prepared aptamers (5 pmol in 10 μ L of 1 x ThermoPol PCR) were incubated with prepared fresh sperm (10⁶ sperm in 250 μ L of selection buffer). Aptamer-sperm incubation was performed for 30 min at room temperature in a light-shielded polypropylene tube. The

sperm and aptamer incubations were then washed once to remove unbound aptamers by centrifugation (400 x g, 7 min, room temperature) and resuspended in 250 μ L of selection buffer.

For flow-cytometry testing, positive, negative, and control, incubations were performed for selection round 7 aptamer pools, and positive and negative incubations only for selection round 9 aptamer pools, with both X- and Y-sex-sorted sperm. As unlabeled control, sperm fluorescence was also measured in absence of aptamers. Washed incubations (250 μ L) were diluted to 1 mL with selection buffer and fluorescence was collected using FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Aptamer FITC fluorescence was detected (round 7: 30000 events; round 9: 10000 events) using argon laser (488nm) with FL1 photomultiplier tube (530/30nm) and was analyzed using CELLQuest Software (Becton Dickinson Immunocytometry systems). Voltage settings (FL1 PMT) were set to locate, and account for endogenous fluorescence, using the unlabeled no-aptamer control sperm population. No population gating was used. Emission intensity data from positive, negative, and control incubation were subtracted from unlabeled no-aptamer control. Differences in population fluorescence distributions between treatments were analyzed by Kolmogorov-Smirnov test.

For fluorescent binding assays, positive, negative, and control, incubations were performed with sex-sorted sperm and aptamer pools from selection rounds 9, 10, and 11. The fluorescence of sperm in absence of aptamers served as unlabeled control. Sperm fluorescence after washing was detected by transferring 50 μ L of 250 μ L incubation

reaction to black 96-well medium-bind microtitre plates (Ultident Scientific, St. Laurent, QC, Canada) and detecting fluorescence with 1.0 sec exposure (excitation filter 485nm, emission filter 535nm) using Victor³ 1420 Multilabel Plate reader (Perkin Elmer, Waltham, MA, USA). For normalization, aptamer raw fluorescence was also measured (5 pmol aptamer in 10 μ L of 1 x ThermoPol PCR buffer, diluted in 250 μ L selection buffer). Fluorescence data from aptamer-sperm incubations was normalized based on X-, Y-, and initial aptamer raw fluorescence and expressed as the fold increase over the unlabeled negative control. Normalized fluorescence data (using data from three rounds as experimental triplicate) was analyzed by analysis of variance (ANOVA) using Tukey test. Direction of aptamer evolution was also inferred from normalized fluorescence data over three rounds by fitting trend line using Excel software (Microsoft® Excel 2000, Microsoft Corporation). Due to lack of inter-round replication of fluorescent assay, no statistical analysis could be performed with respect to the direction of aptamer evolution or trend line.

For fluorescence microscopy, selected X- and Y-aptamer pools from selection round 9, and the initial library, were tested with fresh unsorted sperm to quantify the percent of sperm that bind aptamers. For unlabeled control, sperm were also incubated in absence of aptamers. To concentrate samples, sperm were resuspended after washing in 50 μ L of selection buffer (instead of 250 μ L). For analysis, 10 μ L of post-wash reaction was smeared onto untreated glass microscope slides and allowed to dry for 5 min. Coverslips were mounted with Prolong antifade mounting medium and allowed to cure overnight (Invitrogen, Carlsbad, CA, USA). Four slides for each X-, Y-, initial aptamers, and

negative control, were prepared. Sperm were imaged under fluorescent and bright field with a fluorescence light microscope (DMR, Leica Microsystems, Wetzlar, Germany, excitation filter: 450-490nm) and analyzed using OpenLab (version 4.04) software (Improvision, Coventry, England). Fluorescence images were converted to binary image for presence/absence of fluorescence using software algorithm according to consistent threshold across all samples and merged with bright field image to distinguish fluorescent sperm from non-fluorescent sperm. Four images (one image per slide) for each X-, Y-, and initial library, were analyzed by manually counting all fluorescent and non-fluorescent sperm within image (initial library: 768 sperm; X-aptamers: 728 sperm; Y-aptamers: 872 sperm). Percent of sperm, which bound aptamers above the fluorescence threshold, were analyzed between treatments by analysis of variance (ANOVA).

2.2.5. Aptamer cloning and Sequencing

Selected X- and Y-aptamers from round 20 of the SELEX procedure were amplified a final time using regular PCR and unlabeled primers to produce double stranded aptamers. Amplification was carried out in 25 μ L reactions containing 2.5 μ L of 1x ThermoPol reaction buffer (New England BioLabs, Ipswich, MO, USA), 1.25 units of *Taq* polymerase, 0.4 μ M of unlabeled primers (forward primer 5'-TCCATCTCTTCTGTATGTCGAGATCTA; reverse primer 5'-AATTAACGGAGTCGGTTAGGAGATCTA-3'), 200 μ M of dNTP mix. Amplification reaction was performed using a Biometra TGradient thermocycler with one cycle of 95°C for 4 min, 30 cycles of 95 °C for 30 s, 61.6 °C for 30 s, and 72 °C for 30 s, and a final extension step of 72 °C for 5 min. Double stranded aptamers were purified using

recommended procedures by QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Aptamers were cloned using the pGEM-T Easy system (Promega, Madison, WI, USA) and transformed into DH5 α competent cells per standard procedures (Invitrogen, Carlsbad, CA, USA). For both X- and Y-aptamer pools, 25 plasmids containing inserts were submitted for sequencing (Laboratory Services, University of Guelph). The melting temperatures of the full 90-nucleotide aptamers and the percent of guanine/cytosine nucleotides (%GC) within aptamer random region were calculated using online DNA calculator (Sigma, St. Louis, MO, USA). Differences in the melting temperature and %GC between X- and Y-aptamer groups were analyzed by unpaired t-test.

2.2.6. Position specific aptamer scoring matrix

A position specific scoring matrix (PSSM) scores individual nucleotide positions, assuming independence, based on the overall average nucleotide identity for that position among all sequences in a group and has been used for transcription factor binding site identification (Ben-Gal *et al.*, 2005). A scoring matrix was adapted here for comparing aptamer relatedness within the X/Y groups and termed a position specific aptamer scoring matrix (PSASM). In the PSASM, the score for a particular aptamer was the sum of the position-specific scores across all positions of that aptamer. For aptamer analysis, two scoring matrices were created based on the sequenced X- or Y-aptamers and termed the X- and Y-type matrix, respectively. Aptamer scoring matrices were created by aligning the sequenced aptamers and determining the rate of the four nucleotide (adenine, thymine, cytosine, guanine) identities at each position of the aptamer. The rate of nucleotide identity at a particular position becomes the matrix score for a given

nucleotide in that position. To determine if the selected pools were different, all aptamer sequences (X and Y) were applied to both matrices to calculate scores based on the X- and Y-type matrix. For non-selection controls, a random set 36-nucleotide sequences was obtained and also applied to both matrices. Scores from X-aptamers, Y-aptamers, and random sequences were analyzed within matrix (X-type or Y-type) by analysis of variation (ANOVA) using Tukey test to show differences in aptamer selection outcome.

2.2.7. Identification conserved aptamer motifs

Conserved motifs among selected aptamers were retrieved using Weeder software (version 1.3) (Pavesi *et al.*, 2004; Pavesi *et al.*, 2006). Sequences were searched only on aptamer strand (functional aptamer strand) and the significance of returned motifs was calculated with respect to background frequencies taken from *Homo sapiens* promoter sequences using the Weeder P-value calculator. In addition to motif sequence and score, the location and frequency of each retrieved motif was also recorded.

2.2.8. Aptamer sequence homology to bovine genome

All aptamer sequences (36-nucleotide aptamer region only) were verified against available sequence information for *Bos taurus* (taxid: 9913) using a basic local alignment search tool (NCBI Blastn, Altschul *et al.*, 1990) to identify matches in most recent genomic reference sequence database (build 4.0). Default algorithm parameters for short input sequence were applied. Partial homology matches were considered significant if expect threshold value was less than or equal to 1.0 ($E \leq 1$).

2.2.9. Aptamer secondary structure prediction

Aptamer secondary structures of all sequenced full-length aptamers (17X, 17Y) were predicted using *mfold* DNA folding software (SantaLucia, 1998; Zuker, 2003). Folding predictions were performed for conditions representative of sperm-SELEX experimental conditions (25°C; 10 mM [Na⁺]; 2 mM [Mg⁺⁺]) (Peyret, 2000). The secondary structure tendency (stem or loop) for each nucleotide, of all returned structural isoforms, from all aptamers, was analyzed to show structural differences at each position between X- and Y- aptamers by unpaired t-test.

2.3. Results

2.3.1. Monitoring the selection of X- and Y-aptamer probes to bull sperm.

In-depth aptamer evaluations were conducted in selection rounds 7 through 11 using flow-cytometry, fluorescent binding assays, and fluorescence microscopy, to determine the success of the early rounds of aptamer selection for both the X- and Y-aptamer pools.

Selection round 7 and 9 candidate aptamer pools were tested by flow-cytometry to determine if aptamers had increased binding to the proper sex-type of sperm. Round-7 aptamer binding to X-sperm shows that the X-aptamer pool has higher binding to X-sperm than do the Y-aptamers (Figure 3A). The X-aptamers have only slightly higher binding than the initial library control, however, the Y-aptamers to a greater degree, have lost the ability to bind to X-sperm. Selection round 7 aptamer pool binding to Y-sperm showed that the Y-aptamers unexpectedly had lower binding than both the X-aptamers and initial aptamer library control at this stage of selection (Figure 3B). Furthermore, aptamer binding to Y-sperm was overall significantly lower for all samples than that seen for X-sperm binding. Selection round 9 aptamer binding to X- and Y-sperm again showed that the X-aptamers outperformed Y-aptamers in binding to X-sperm (Figure 3C). In this round however, the Y-aptamers had higher binding to Y-sperm, compared to the X-aptamer pool, as would be expected (Figure 3D). Initial library control was not used in this evaluation so direction of selection cannot be inferred. Curve shift between X/Y aptamers, in all tests, were found to be significantly different by Kolmogorov-Smirnov statistic ($P \leq 0.001$).

Figure 3. Flow-cytometry binding analysis of selected aptamer pools to sorted sperm. Selection round 7 analysis for X-sperm (A) and Y-sperm (B) based on 30,000 events subtracted from negative control. Selection round 9 analysis for X-sperm (C) and Y-sperm (D) based on 10,000 events subtracted from negative control. All curve shifts between X/Y-aptamers were found to be significantly different from each other by Kolmogorov-Smirnov statistic ($P \leq 0.001$).

The fluorescent binding assay measures aptamer fluorescence emitted from sperm after incubation (and sperm washing to remove unbound aptamers) to determine ability of the selected X-, Y-, and initial aptamers, to bind sorted X/Y sperm (Figure 4,5). The fluorescent binding assay was performed using selected aptamer pools from selection rounds 9, 10, and 11. For testing with X-sorted sperm, when the data from the three rounds was combined, the binding assay found that the X-aptamer pool produced the highest fluorescence fold-increase over unlabelled sperm (1.61x) compared to the Y-aptamer pool (1.15x) and the initial aptamers (1.14x), however, the increase in fluorescence was found to be not significant by analysis of variance using Tukey test (Figure 4A). The lack of significance was caused by large variance within aptamer treatments (but not with initial library control) across the three selection rounds due to evolving aptamer pools (variance: X-apt =0.087; Y-apt =0.025; initial apt =0.001). To infer direction of aptamer evolution the data were plotted over the three individual rounds, and a trendline fitted (Figure 4B). The trend in aptamer evolution could not be statistically analyzed, but appeared to indicate that the X-aptamer pool was increasing in binding ability to X-sperm over these three rounds while the Y-aptamer pool was decreasing in the ability to bind X-sperm, resulting in apparent large differences among treatments.

For testing with Y-sorted sperm, when the data from the three rounds was combined, the binding assay found that the Y-aptamer pool produced the highest fluorescence fold-increase over unlabelled sperm (1.78x) compared to the X-aptamer pool (1.32x) and the initial aptamers (1.07x). The Y-aptamer fold-increase in fluorescence was found to be

significant by analysis of variance using Tukey test compared to the initial aptamers but not compared to the X-aptamers ($p < 0.05$) (Figure 5A). Again large variance was observed, in particular within the Y-aptamer treatment, across the three selection rounds (variance: X-apt = 0.060; Y-apt = 0.145; initial apt = 0.021). To infer direction of aptamer evolution the data were plotted over the three individual rounds, and a trendline fitted (Figure 5B). The trend in aptamer evolution appeared to be (again, no statistical analysis was possible) that the Y-aptamer pool was increasing in binding ability to Y-sperm over these three rounds while the X-aptamer pool and the initial aptamers were not notably evolving to Y-sperm.

Figure 4. Fluorescence binding assay of aptamer pools to sorted X-sperm. Fluorescence expressed as the fold-increase over unlabeled negative control sperm was measured for X-aptamers, Y-aptamers, and initial library, from selection rounds 9, 10, and 11. Data was analyzed using selection rounds as replicate (n=3) by analysis of variance (ANOVA) using Tukey test (**A**). No significant differences were found between treatments. Error bars indicate standard error of mean. Direction of aptamer evolution was also inferred from fluorescence data over the three rounds by fitting trend line (**B**); nature of the data precluded statistical analysis of the direction of aptamer evolution (trendline).

Figure 5. Fluorescence binding assay of aptamer pools to sorted Y-sperm. Fluorescence expressed as the fold-increase over unlabeled negative control sperm was measured for X-aptamers, Y-aptamers, and initial library, from selection rounds 9, 10, and 11. Data was analyzed using selection rounds as replicate (n=3) by analysis of variance (ANOVA) using Tukey test (**A**). Bars with different annotation were found to be significantly different ($p < 0.05$). Error bars indicate standard error of mean. Direction of aptamer evolution was also inferred from fluorescence data over the three rounds by fitting trend line (**B**) nature of the data precluded statistical analysis of the direction of aptamer evolution (trendline).

The selected aptamer pools were tested with regular unsorted, unfrozen, sperm to determine if aptamers could detect the 50:50 ratio of X- to Y-sperm in semen. The percentage of sperm that bound selection round 9 X- and Y-aptamers, and an initial library control, was quantified by fluorescence light microscopy. None of the samples displayed the expected 50% binding pattern. The X-, Y-, and initial aptamers bound 34% (n=728), 31% (n=872), and 36% (n=768) of the sperm, respectively (Figure 6A). No significant difference between treatments was found by analysis of variance ($p=0.37$). Binding of selected aptamers and the initial aptamer was found primarily in the post-equatorial region of the sperm head (Figure 6 B-E).

A

B

C

D

E

Figure 6. The percent of sperm in unsorted semen that bind aptamers either before selection (initial library) or after 9 rounds of selection to X-sperm or Y-sperm, as quantified by fluorescence light microscopy, did not differ (ANOVA, $p=0.37$). Numbers above columns indicate total number of sperm counted per treatment (A). Representative fluorescence and overlay images used for counting from X-aptamers (B), Y-aptamers (C), initial aptamers (D), and no aptamer negative control (E). (Scale bar: 25 μm)

2.3.2. Aptamer cloning and sequencing

After twenty rounds of selection the candidate X- and Y-aptamer pools were cloned into TA vector and sequenced to identify any conserved sequence(s) or structure(s). For both X- and Y-aptamer pools 25 colonies were sequenced, resulting in characterization of 34 full-length aptamer sequences (17 X, 17 Y) and 16 truncated aptamer sequences (Table 2). No sequenced aptamers were found to have the exact same sequence. The truncated aptamers varied in length and were not found to contain any conserved features or nucleotide repeats. The melting temperature (average: X-apt =91.3 °C; Y-apt =91.1 °C) and the percent guanine/cytosine (average: X-apt =60.2%; Y-apt =61.4%) of sequenced full-length aptamers was found to be not significantly different between X- and Y-aptamers by unpaired t-test.

Table 2. Aptamer length, melting temperature, and rate of guanine/cytosine nucleotides in aptamer random region, among 17 X- and 17 Y-aptamer full-length sequences.

Seq ID	Length	T _m (°C)	%GC
X-aptamer #1	90	91.03	64
X-aptamer #3	90	90.74	56
X-aptamer #4	89	90.28	56
X-aptamer #6	90	90.33	61
X-aptamer #7	90	92.11	64
X-aptamer #8	90	91.18	58
X-aptamer #9	91	89.67	47
X-aptamer #10	90	92.41	64
X-aptamer #11	90	90.29	53
X-aptamer #12	90	93.16	75
X-aptamer #14	90	93.93	67
X-aptamer #15	90	87.94	53
X-aptamer #18	90	90.94	64
X-aptamer #19	90	88.28	44
X-aptamer #20	90	93.02	64
X-aptamer #21	90	93.52	69
X-aptamer #22	90	93.32	64
Y-aptamer #1	93	88.15	56
Y-aptamer #2	90	88.68	50
Y-aptamer #3	91	91.02	58
Y-aptamer #7	90	92.33	67
Y-aptamer #8	90	92.03	67
Y-aptamer #10	90	89.62	56
Y-aptamer #11	90	92.06	61
Y-aptamer #12	90	89.6	56
Y-aptamer #14	90	91.15	61
Y-aptamer #15	90	89.81	61
Y-aptamer #17	90	91.9	64
Y-aptamer #19	90	90.62	61
Y-aptamer #20	90	94.29	72
Y-aptamer #21	90	89.82	56
Y-aptamer #22	90	93.94	75
Y-aptamer #23	90	91.25	56
Y-aptamer #24	91	91.95	67

2.3.3. Aptamer characterization: Position specific aptamer scoring matrix

An X-type and Y-type position-specific aptamer scoring matrix was used to score the aptamers based on sequence conservation within the two candidate pools. When all 17 X- and 17 Y-aptamers, and a set of 17 random sequences, were scored according to the X-type scoring matrix (X-PSASM), 13 out of the 14 top scoring aptamers were found to be X-aptamers with only one Y-aptamer (Y-aptamer #22) scoring in the top 14. Likewise, when all 17 X- and 17 Y-aptamers, and a set of 17 random sequences, were scored according to the Y-type scoring matrix (Y-PSASM), only one X-aptamer (X-aptamer #6) scored within the top 14 scoring aptamers. When X-PSASM scores were plotted against Y-PSASM scores the X/Y aptamer types and a group of 17 random sequences cluster into distinct groups (Figure 7). All X-aptamer sequences score above the median score for the X-PSASM (median =1006; range of X-aptamer scores: 1024-1300), while all but two Y-aptamer sequences score above the median score for the Y-PSASM (median =1025; range of 15/17 Y-aptamer scores: 1053-1324, two lower scores =982, 1018). The average scores of the X-, Y-, and random sequence set, from the two matrices, were compared by analysis of variance using Tukey test. For the X-PSASM, the average X-aptamer score (score =1166) was significantly higher than both the average Y-aptamer score (score =986) and the average score of the random sequence set (score =956) ($p<0.05$). For the Y-PSASM, the average Y-aptamer score (score =1144) was not significantly higher than the X-aptamer score (score =983) but was significant with respect to random sequence set (score =950) ($p<0.05$).

Figure 7. Position specific aptamer scoring matrix. Selected aptamer sequences and a random set of 36-nt sequences scored using X-PSASM (x-axis) and Y-PSASM (y-axis). Crosshairs represent average scores for each aptamer type showing standard error of mean from both X-PSASM and Y-PSASM. Data were analyzed by analysis of variance (ANOVA) using Tukey test. Error bars with different annotation (a, b, for X-PSASM; c, d, for Y-PSASM) were significantly different within same matrix type. Quadrants lines (grey) represent median score of PSASMs.

2.3.4. Aptamer characterization: Aptamer secondary structure prediction

Aptamer secondary structure(s) were predicted for all candidate aptamer sequences to identify conserved structural regions that may be involved in aptamer folding or binding. As individual aptamer sequences often had several predicted isoform structures, all computed structural isoforms were combined to determine the likelihood for each position of all aptamers to exist as a loop (unpaired) or stem (paired) structure (e.g., Figure 8). In total, analysis found 65 X-aptamer, and 76 Y-aptamer, isoform structures from the 17 X and 17 Y aptamer sequences, respectively. Secondary structure annotations for X- and Y- aptamers were pooled to compare the average secondary structure tendency over the length of the X-and Y-aptamers (Figure 9). Comparison of the aptamer secondary structures found that the fixed primer regions had similar structure between the X- and Y-aptamers, however, within the aptamer region, the X/Y aptamer types had more divergent structures. In total, 17 of the 90 positions within the aptamers (15 occurring within 36-nucleotide aptamer random region) were found to have significantly different secondary structure tendency between the predicted X- and Y-aptamers. Specifically, the Y-aptamers show strong tendency to form loop-structures around positions 44, 47, 55 and 61, while the X-aptamers show tendency to adopt a different structure around these positions.

Figure 8. Isoform secondary structure predictions for X-aptamer #1.

Figure 9. Average secondary structure tendency over length of X- and Y-aptamers. The percent of aptamer isoforms that adopt loop structure is shown on Y-axis, with a score of 100% indicating that aptamer secondary structure is in loop (unpaired) formation in all predicted isoforms for all aptamers. Score of 0% indicates aptamer secondary structure is in stem (paired) formation. Lines at positions 27 and 63 indicate primer-random region division (X-axis). Average secondary structure tendency is compared between predicted X- and Y-aptamer structures using unpaired t-test at each nucleotide position. Error bars representing standard error of mean are shown for all positions found significantly different between X- and Y-aptamer predictions ($p < 0.05$).

2.3.5. Aptamer sequence homology to bovine genome

All aptamer sequences (36-nucleotide aptamer region only) were verified against available sequence information for *Bos taurus* using a basic local alignment search tool (blastn) to determine if aptamers have homology to any areas of genomic DNA. No perfect homology (36/36 nucleotides) was identified for any of the selected aptamers to the genome database. However, some incidences of significant partial matches were identified in both X- and Y-aptamer groups ($E < 1$). Among 17 X-aptamers, 8 sequences had significant partial matches to genome (X-aptamers #3, #4, #6, #8, #9, #15, #18, #22). Likewise, among 17 Y-aptamers, 6 sequences had significant partial matches to genome (Y-aptamers #1, #3, #7, #21, #22, #23). The highest homology was found in X-aptamers #6 and #8, which had regions of 22/23 and 19/19 homology with chromosomes 4 and 8, respectively ($E = 0.15$).

2.3.6. Aptamer characterization: Identification of conserved aptamer motifs

Conserved motifs among selected aptamer pools were identified using Weeder software. An 8-nucleotide X-aptamer motif (score: 1.77; $p < 0.001$) was identified in 9 out of 17 X-aptamers, with X-aptamers #10, #12, and #22, having two copies of the motif, and X-aptamer #20 having 4 copies of the motif. The distribution of the X-motif was predominantly toward the 3'-third of the randomized aptamer region (start position: range = 35-56; median = 49) (Table 3). Only X-aptamer #10 contains the consensus motif, however, X-aptamer #20 contains four statistically relevant copies of this X-motif - three of which occur as tandem repeats. The consensus X-motif was also unexpectedly found in Y-aptamers #2 and #22 as a 6/8 position match.

A 10-nucleotide Y-aptamer motif (score: 1.52; $p < 0.001$; containing an 8-nucleotide redundant root motif) was identified in 11 out of 17 Y-aptamers, with only Y-aptamer #22 having two copies of the motif. The distribution of the Y-motif was predominantly toward the middle-third of the randomized aptamer region (start position: range = 28-54; median = 41.5) (table 4). The consensus Y-motif was found in X-aptamer #1 as an 8/10 position match; and in X-aptamers #6 and #10 as 7/10 position match.

Table 3. Distribution of X-aptamer motif among full length X-aptamers. Match percentage score represents nucleotide percent match to consensus 8-nucleotide motif weighted for position degeneracy.

Seq ID	Position range (nt.)	Match percentage
X-aptamer #1	40-48	94.01
X-aptamer #4	52-60	93.77
X-aptamer #9	56-64	92.90
X-aptamer #10	46-54	90.93
X-aptamer #10	54-62	100.00
X-aptamer #12	37-45	91.17
X-aptamer #12	56-64	95.74
X-aptamer #18	47-55	95.43
X-aptamer #19	36-44	91.24
X-aptamer #20	35-43	90.79
X-aptamer #20	43-51	95.74
X-aptamer #20	51-59	88.40
X-aptamer #20	55-63	86.99
X-aptamer #22	49-57	92.66
X-aptamer #22	56-64	93.18

Table 4. Distribution of Y-aptamer motif among full length Y-aptamers. Match percentage score represents nucleotide percent match to consensus 10-nucleotide motif weighted for position degeneracy.

Seq ID	Position range (nt)	Match percentage
Y-aptamer #1	36-46	87.67
Y-aptamer #2	53-63	92.04
Y-aptamer #3	41-51	100.00
Y-aptamer #7	31-41	93.40
Y-aptamer #8	32-42	87.86
Y-aptamer #15	28-38	96.00
Y-aptamer #19	42-52	87.67
Y-aptamer #20	45-55	89.95
Y-aptamer #21	54-64	90.29
Y-aptamer #22	46-56	90.24
Y-aptamer #22	50-60	90.24
Y-aptamer #24	41-51	90.72

2.4. Discussion

Aptamers were shown here to be useful tools for biomarker discovery in sperm. The aptamer selection approach developed here successfully selected unique X- and Y-aptamer types, with different functional and physical properties, to X- and Y-sperm, respectively. The described aptamer selection approach could readily be applied for development of aptamer(s) to other sperm features.

Aptamer functional properties were evaluated at the midpoint of selection using flow cytometry, fluorescent binding assays, and fluorescent-light microscopy to verify that selection was being achieved as hypothesized. The physical properties of the selected aptamers were evaluated post-selection by sequence analysis using software-based approaches.

Aptamer functional testing used aptamer fluorescence, to detect and quantify the fluorescence emitted by the aptamers bound to sperm, and thereby partially characterize aptamer-sperm interaction. The fluorophore FITC was bound to the selected aptamers, exposed to sperm, and the fluorescence intensity was equated to the ability of aptamers to remain bound to sperm after stringent washing, which was considered indicative of specific target binding and intrinsic aptamer function. The detected fluorescence was assumed to be from FITC-labeled sperm-bound aptamers only, and not free-FITC molecules, or other fluorescent particles. Unlabeled sperm were used to account for endogenous sperm fluorescence in absence of treatment. To estimate non-specific

binding, the initial library and/or the opposite selected pool were used as negative (non-sex chromosome-specific) controls during testing.

The fluorescence emitted by individual sperm measured by flow-cytometry demonstrated that the sex-sorted X- and Y-sperm had significantly higher fluorescence when exposed to their respective selected X- and Y-aptamer pools, than did sperm exposed to either the opposite pool or the initial unselected library. Binding of more of the selected aptamers over the initial library indicates that selection has taken place and aptamers were evolving toward the ability to bind the proper sex of sperm. Binding of more of the selected aptamers over the opposite selected pool indicates that binding characteristics of X- and Y-aptamer pools were different, consistent with the two pools each having been selected to different targets. The degree of selection relative to initial library shows that the counter-selected pool has undergone more evolution (losing ability to bind certain sex of sperm) than the positively selected pool (Figure 3A,B). This would be expected due to the double negative selection, compared to the single positive selection that was used in each selection cycle. Both the curve shape and magnitude of the shift between pools (Figure 3) were consistent with published reports of selecting aptamers to free cells using similar methods (Shangguan *et al.*, 2006).

The fluorescence binding assay parallels the flow-cytometry functional testing by again quantifying aptamers bound to sperm as a measure of specific target binding and intrinsic aptamer function. The fluorescence assay gives a snapshot of the total fluorescence emitted from sperm population after aptamer binding under more realistic conditions than

flow-cytometry, however, does not discriminate for non-sperm-bound aptamer fluorescence (ie: aptamers bound to contaminating cells, or free-floating aptamers remaining after washing). As with the flow-cytometry experiment, sex-sorted X- and Y-sperm had higher fluorescence when exposed to their respective selected X- and Y-aptamer pools from selection rounds 9, 10 and 11, than did sperm exposed to the opposite pool or the initial unselected library (Figure 4A, 5A). Although, the expected pattern of increased binding was seen for both X- and Y-sperm, the increase in fluorescence was only found to be significant in testing with Y-sperm, between the Y-aptamers and the initial unselected library, due to large variance within treatment (aptamer type) over the three selection rounds (experimental replicate). This variance would be expected only if aptamer pools were actively evolving over the course of the three selection rounds. To infer how aptamer pools were evolving, and in what direction, over selection rounds 9-11, the data were split and then fitted to a trendline (Figure 4B, 5B). The trendline illustrates how the changing aptamer libraries account for observed variation within treatment (aptamer type) due to progressive aptamer evolution as would be expected. The trendline shows that the X-aptamer pool was progressively increasing in binding ability to X-sperm over the course of the three rounds, while the Y-aptamer pool was progressively losing its ability to bind X-sperm due to negative selection. Similarly, the Y-aptamer pool was progressively increasing in binding ability to Y-sperm over the three rounds. The trend in the unselected initial aptamer library over the three rounds as expected does not appreciably change (confirming no selection was occurring) providing confidence in the strength of the fluorescence assay experimental approach. The trendline holds no statistical power (no inter-round replication) however, does show that the

selected X- and Y-aptamers types were progressively being selected to two different targets.

Fluorescence microscopy further assessed aptamer function, quantifying the percent of aptamers that bind unsorted sperm (Figure 6). Since unsorted semen is assumed to contain an equal ratio of X- to Y-sperm, selected sex specific aptamers should bind to only half of the sperm. This was an important indicator of aptamer function because the physical stress of sperm sorting might damage sperm to the point that aptamers selected to sorted sperm features might not function with regular unsorted sperm. Particularly, if the 'degree of damage' was different between the X- and Y-sorted sperm this may be a stronger sorting index than any sex-specific molecular differences. Therefore normal untreated fresh sperm were used occasionally for positive selection to try to overcome any potential inadvertent selection to damaged sperm. To test if aptamer binding characteristics seen with sorted sperm would hold for unsorted sperm, a simple fluorescence microscopy test counted the ratio of fluorescent sperm after exposure to labeled aptamers, and compared that to the expected 50:50 model. Slightly less than half bound the initial library and the selected X/Y-aptamer pools (initial =36%, X-apt =34%, Y-apt =31%). The percent of sperm that bound the X- or Y-aptamers did not differ significantly, which would be expected, as both aptamer types should bind half of the sperm. The percent bound by each aptamer is slightly lower than the expected 50%, possibly due to different sub-populations within semen capable of binding aptamers (ie: live vs. dead). Unexpectedly, the initial library used here as a negative control bound a similar percentage (no significant difference from either X- or Y-selected aptamers) of

the sperm as the selected aptamers (Figure 6). Unselected aptamers were hypothesized to bind to either all, or none, of the sperm, so the limited binding could mean that only a sub-population of regular sperm (approximately 30%-40%) can bind aptamers regardless of selection. It is also possible that within subpopulations of sperm that bind aptamers, ~50% are individually binding more selected aptamers per cell, which is consistent with the 1.5 fold fluorescence increase found with selected aptamers in the fluorescent assay (Figure 4,5). This fluorescence intensity would not be detectable visually on the microscope. Of course, a different sub-population could be binding to the initial aptamers than was binding to either selected aptamers.

The sort-purity of sorted sperm used during selection rounds 1-10 was not ideal for optimal selection of sex-specific aptamers. The lower purity of the Y-sorted sperm samples (80-85% Y-sperm) might have resulted in less efficient selection. This might have resulted in some carry-over of X-sperm binding aptamers into Y-aptamer pool. Higher quality sorted sperm (92% X-sperm, 95% Y-sperm) was obtained and used in second half of selection rounds, which should have resulted in the final aptamers obtained after the 20 selection rounds being of at least equal, and probably higher, specificity than those tested at rounds 9-11.

Although aptamers were clearly binding sperm, it was unclear if the binding specifically reflected the X/Y genome. Several sperm head proteins can bind DNA (Wu *et al.*, 1990; Zani *et al.*, 1995; Lavitrano *et al.*, 1997). The 30-40% of sperm with bound aptamers was very similar to previous evidence showing that 30-35% of rabbit spermatozoa bound

labeled SV40 DNA (Brackett *et al.*, 1971), while 39-78% of mouse sperm bound foreign pSV2CAT plasmid DNA (Camaioni *et al.*, 1992). In pig, from 0.26%-0.3% (Castro *et al.*, 1990) to 78% (Camaioni *et al.*, 1992) to 90% of sperm were shown to bind DNA depending on the thoroughness of seminal plasma removal (Lavitrano *et al.*, 2003). The binding pattern of aptamers to the post-acrosomal region indirectly confirms non-specific binding, since it was reminiscent of the pattern seen previously for binding of non-specific exogenous DNA to sperm (Brackett *et al.*, 1971; Spadafora, 1998). If the binding to these fresh unsorted sperm was non-specific, it would indicate that selecting aptamers to two sex-sorted populations was selecting for features associated with, or resulting from, the sorting process, and not the sex chromosome. However, the significant differences observed between X- and Y-aptamers, from flow-cytometry and fluorescence assay, might also indicate a combination of specific and non-specific binding. It is possible that specific target sites become saturated by large number of available aptamers resulting in a large number of selected aptamers binding non-specifically. In addition, the selected X and Y aptamers used for this experiment still constitute a complex pool and the proportion of specific aptamers was not known. Aptamers also might not have folded properly under conditions used for the microscopy due to different ionic strength or temperature increase during microscopic observation. For example, we recently found a strict requirement for specific ionic strength for proper aptamer folding (data not shown). It is clear that there are many potential cases that might lead to observed non-specific binding. At the same time the better binding of the selected aptamers over the initial library determined by flow-cytometry and fluorescent assays seems to indicate that under some conditions selected aptamers were able to differentiate two sperm populations.

After 20 selection rounds the pools were cloned and sequenced to characterize the physical properties of the selected aptamers. From 50 colonies containing aptamers, 34 (17X and 17Y) were found to contain full-length aptamers. Truncated aptamers were also present, at an equal rate in the two aptamer pools, which suggests that the truncation mechanism was independent of the selection process. Truncated aptamers likely arise during PCR, or replication within bacteria, when the enzyme skips portions of the sequence due to strong secondary structures or long nucleotide repeats (Viswanathan *et al.*, 1999). The truncated aptamers varied in length and were not found to contain any conserved features.

Analysis of raw sequence data showed that among the full-length aptamer sequences (17 X, 17 Y) none were found to be exactly the same, which could indicate that sequencing was not saturated and that pools were still complex. When the whole cell approach was used to develop aptamers in other studies that also used 20-21 cycles, multiple aptamers were also found after sequencing (Daniels *et al.*, 2003; Shangguan *et al.*, 2006) and only ~10% of 163 sequenced clones were found to be homologous (Daniels *et al.*, 2003). It is therefore possible that sequencing only 17 X-aptamer and 17 Y-aptamer clones is not sufficient to identify homologous sequences in the final pool. The sperm-SELEX scheme was designed to exclude ~75% of aptamers during each selection round using positive and negative selection. Mathematically, if the selection pressure stayed the same, this would result in approximately 450 unique aptamers remaining after 20 selection rounds from the 200 pmol used initially. Theoretically, 25 rounds of selection with 75%

exclusion would have been required to attain a single molecule. However, as selection pressure is increased in later rounds by decreasing the amount of added aptamers to one pmol with 1000-fold excess of competitor DNA, the elimination of aptamers reaches a plateau. Indeed, in latter rounds the aptamer exclusion was observed to be much lower, as indicated by comparing the amount of recovered aptamers to the amount of aptamer used in each selection round, presumably because surviving aptamers were able to overcome selection pressure. This would suggest that aptamers were successfully being selected. More selection pressure could have been implemented into SELEX system; however, the risk of inadvertent elimination of desired aptamers and selection to other aptamer characteristics is increased when selection pressure is very high. Therefore we hypothesize that the average amount of aptamers excluded in each round was likely much lower in later rounds resulting in more unique aptamers present after 20 rounds of selection and subsequent cloning.

The apparent complexity of final aptamer pool may question if SELEX was successful at selecting two different aptamer populations. The fact that no two aptamers were found to be exactly the same does not necessarily mean that the selection was incomplete as aptamers with different sequences can retain a common structure and function. Software based approaches were used to compare sequence conservation, motifs present, and the secondary structure, between sequenced X- and Y-aptamers to determine if selected pools were different from each other indicative of selection to two unique targets on X- and Y-sperm.

A position-specific aptamer-scoring matrix (PSASM) was applied to the unaligned 36-nucleotide aptamer random regions to determine if the selected X- and Y- pools differed from each other as an indicator of successful selection to two different targets. If aptamer selection had failed completely the PSASM would show no significant difference between the X- and Y-aptamer pools. In addition to the selected 17 X- and 17 Y-aptamer sequences, a random set of 17 sequences was used as an unselected control group and applied to the matrix. When X/Y-PSASM scores were plotted against each other the X-aptamers, Y-aptamers, and random set of sequences, remain clustered together as three distinct populations. For the X-PSASM, the average score for X-aptamers was found to be significantly different from both the Y-aptamers and the random set of sequences. This shows that the X-aptamers as a group were significantly unique, indicative of selection to a specific target. For the Y-PSASM, the average score for Y-aptamers was found to be significantly different from the random set of sequences but not from the X-aptamers. This shows that selection has taken place with the Y-aptamer group, but the group as a whole was not unique. This was perhaps due to the larger amount of contamination in the Y-sorted sample (5-20% X-sperm contamination) used during the first half of the selection compared to the X-sorted sample resulting in less specific Y-aptamers.

It seems unlikely that X- and Y-sperm would have multiple different surface proteins, as previous research has found no single molecular difference (Hendriksen, 1999). As such, despite different sequences, the majority of aptamers from the same pool might be expected to bind to a single target site. Although aptamers used for selection are 90-nucleotides in length, the functional region of an aptamer is typically between 8-20

nucleotides, therefore identifying these smaller motifs is important when characterizing aptamers. It is possible that while conserved sequences are present in divergent aptamers they might be small and located in different positions therefore would not be identified by simple sequence comparison. Even a 1-2 nucleotide shift would degrade the conservation and would make it impossible to detect the sequence conservation by simple alignment. It is also possible that a motif might be degenerate, with some positions strongly conserved and some not conserved at all. The Weeder program (version 1.3) which was designed to find novel transcription factor binding sites conserved in a set of related genes (Pavesi *et al.*, 2004; Pavesi *et al.*, 2006), was applied here to the aptamer sequences to identify conserved motifs among the related X- and Y-aptamers. The Weeder software applies a consensus-based (or pattern-driven) algorithm that identifies conserved regulatory sites in given sequences by identifying 6-15 bp sequences which show a higher level of conservation as compared to the rest of the surrounding sequence and scores the motifs according to a statistical measure of significance. The Weeder program has been shown to outperform similar available motif discovery tools (Tompa et al, 2005). In our pools, the Weeder program identified an 8-nucleotide motif (with no degenerate motifs) among 9 of the X-aptamer sequences, and additionally provided 6/8 position matches in Y-aptamers #2 and #22. Y-aptamer #22 was an anomaly, as this aptamer was also found to be the highest scoring Y-aptamer on both the X-PSASM and the Y-PSASM, and therefore perhaps has been selected to both X- and Y-sperm. The Weeder program also identified a 10-nucleotide motif (and an 8-nucleotide redundant version of the motif) among 11 of the Y-aptamer sequences, and additionally provided an 8/10 position match in X-aptamer #1, and 7/10 position matches in X-aptamers #6 and #10. As with Y-

aptamer #22, X-aptamer #6 was not surprising to have the consensus Y-motif as this aptamer scored 7th highest of the 51 sequences (highest of all X-aptamers) on the Y-PSASM. Importantly, there was no incidence of perfect matches between of X- or Y-motifs in X- or Y-aptamer sequences, respectively. The location of the X-motif within the X-aptamers was not conserved, however, none were found in the 5'-third of aptamer region indicating that location towards 3'-half of aptamer region might be important to aptamer function. This might be due to interference by relatively large FITC-fluorochrome at the 5'-end of aptamer, perhaps by steric hindrance, or by influence from one of the fixed primer regions. Aptamer function was abolished when the FITC was exchanged for the larger Cy-5 fluorochrome indicating that spatial interference might occur with aptamers (data not shown). The location of the top scoring Y-motif within Y-aptamers was not well conserved.

Sorting sperm by flow-cytometry causes membrane damage; making it more likely that aptamer selection using sex-sorted sperm might result in aptamers penetrating sperm membrane and binding directly to complementary genomic DNA (Spinaci *et al.*, 2005; Moce *et al.*, 2006). If aptamers bind directly to sex chromosomes, sex-specific aptamer binding might be achieved, however, this mode of binding is unlikely to be useful for attaining the goal of sperm sexing in live, membrane intact, sperm. Aptamer sequences (36-nucleotide random region only) were verified against available sequence information for *Bos taurus* using a basic local alignment search tool (blastn) to identify homology to genomic DNA. No perfect, or near perfect, homology was found in any of the selected aptamer to the genome database. This suggests that the selected aptamer sequences were

not a result of inadvertent amplification of genomic DNA during aptamer amplification and were in fact true selected aptamer sequences. However, partial matches below significance threshold were found in 8 X-aptamers and 6 Y-aptamers to various regions of genome. Partial matches can be in part attributed to chance occurrence given the size of the *Bos taurus* genome (458,661,494 letters) and the relatively short matches (~10-15 nucleotides). For example, a 10-nucleotide motif would be expected to occur by chance at a rate of 43 times in the *Bos taurus* reference genome database. None-the-less with the wide occurrence of partial matches among some selected aptamer to the *Bos taurus* genome it is possible that some aptamers are selected for by binding to the genomic DNA during both negative and positive selection and might not have been selected for by binding to a specific ligand or for sex-specificity. However, the sperm genome is highly condensed in mature sperm therefore these genomic DNA complementary motifs may be inaccessible to aptamers and not play a role in aptamer binding whatsoever.

Aptamer binding is conventionally dependent on the formation of stable, unique, 3D-structures. Full-aptamer structures can be derived by NMR spectroscopy; however, these experiments are very expensive and time consuming (Zhou *et al.*, 1999; Robertson *et al.*, 2000; Warren and Moore, 2001; Clore and Kuszewski, 2003; Mani *et al.*, 2007).

Secondary structure software predictions, based on energy minimizing methods, can quickly provide a good idea of aptamer structure in terms of stems, loops, and bulges. Consensus aptamer motifs are often found in stem-loop structures (Jiang *et al.*, 1997; Horn *et al.*, 2004; Lee *et al.*, 2005; Stoltenburg *et al.*, 2007a); therefore combination of

secondary structure predictions with aptamer motif locations can be valuable in characterizing aptamers.

Comparison of the average X- and Y-aptamer secondary structure tendency highlights a region of interest from positions 40-60 where the average X- and Y-aptamer structures were noticeably different. In this region, the average X-aptamer has tendency for loop-stem-loop structure at positions 50, 55, 60, respectively. Interestingly, this was also the region where the consensus top scoring X-aptamer motif was most often identified among X-aptamers suggesting this region might be the location of aptamer-ligand interaction. The average Y-aptamer secondary structure in position 47 has tendency for loop structure in 89% of all predicted Y-aptamer isoforms which was very strong support for consistent secondary structure (at least at this position). This highlights how aptamer secondary structure can be very well conserved despite apparent degeneracy in aptamer sequence. Interestingly, the average secondary structure of Y-aptamers seems to shift towards paired (stem) immediately after position 47 implicating this loop-stem region as potentially important to Y-aptamer shape.

Based on *in silico* aptamer sequence analysis, despite divergent primary sequences, the X-aptamer group appears to have been selected with a strong consensus motif in a specific region of the aptamer. Although no inference can be made on the identity of the target of the X-aptamers it can be concluded that the selection process was successful and that X-aptamer pool has been selected to a specific single target. In particular, two X-aptamers have emerged as interesting candidates for further testing. X-aptamer #20

contains four copies of the consensus X-motif – three of which appear as tandem repeats. Also, X-aptamer #12 scored highest of all selected aptamers in the X-PSASM and 4th lowest of all selected aptamers on the Y-PSASM. Furthermore, X-aptamer #12 contains two copies of the consensus X-motif both of which occur in appropriate loop-stem-loop structure. The Y-aptamer group was found to contain a highly conserved secondary structure at position 47, which suggests that selection toward a common feature was successful. The Y-aptamer group does contain a consensus motif, however, location of this motif appears to be randomly distributed across aptamer. This might suggest that the Y-aptamers do not function with conventional aptamer-shape-dependant binding and might be stabilized into specific structure after binding or were binding directly to genomic DNA or non-specifically.

2.5. Conclusion

Two concurrent sperm-SELEX procedures were used here with the goal of developing sex-specific aptamer probes to bovine X- and Y-sperm. Two key points must be considered when interpreting the results to evaluate achievement of this goal: 1) did the sperm-SELEX procedure select aptamers to bull sperm; 2) were the selected aptamers sex-specific for X- and Y-sperm. Flow cytometry, fluorescent binding assays, fluorescence microscopy, and physical characterization by scoring matrices, secondary structure predictions, and identification of consensus motifs, all demonstrate occurrence of aptamer selection in both the X- and Y-aptamer pools. Results from the middle of SELEX using flow cytometry and fluorescent binding assays suggest that aptamers pools

might be displaying sex-specific binding, however, more testing is required to confirm the sex-specificity. In particular, more testing using individual aptamers is required with unsorted sperm to determine if aptamer function is limited to use with sorted sperm. Moving forward, a method capable of determining the sex of single sperm will need to be developed for testing aptamer sex-specificity (chapter 3). With the available aptamer sequences, more thorough testing using a monoclonal aptamer approach, using the candidate aptamers identified here, can be conducted to fully characterize individual aptamers and the dynamics of aptamer-sperm interaction (chapter 4).

CHAPTER 3: DEVELOPMENT OF SINGLE SPERM SEXING BY AMELOGENIN NESTED POLYMERASE CHAIN REACTION

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3.1. Introduction

Resolving the sex chromosome type of individual sperm, or the X:Y ratio in semen, is of particular interest in many reproductive disciplines to evaluate the role of the sex-chromosomes on sperm processes, or to determine the success of a sperm sexing procedure. Sperm sexing, the physical separation of X- and Y-sperm using flow cytometry, has developed into a mainstream technique in animal breeding programs for sex-selection (Johnson, 2000; Garner, 2001; Garner, 2006). Ongoing process development, and quality control for production, relies on effective means to determine the purity of sorting. Currently, the purity of sorted semen is determined by sort re-analysis wherein the sorted sperm is re-analyzed using the same flow cytometer technology. This type of sort purity evaluation is convenient, and has been shown to be quite accurate (Welch and Johnson, 1999), however, uses the same instrumentation, therefore, would carry-over any errors that have resulted in improperly sorted sperm. Sort re-analysis also requires a relatively large number of sperm to be used (~50000 sperm) (Welch and Johnson, 1999). A sex-ratio determination method for pooled bovine semen, by quantitative real-time PCR, using TaqMan probes to the X-chromosome proteolipid protein gene (PLP), and the Y-chromosome male sex determination gene (SRY), has

been reported as an accurate alternative to sort re-analysis (Parati *et al.*, 2006), however, this method still requires a relatively large test sample of 4000 to 5000 sperm.

When developing a novel sperm sexing technique the number of sexed sperm might initially be small requiring application of single sperm sex testing to check the efficiency of sexing. For single sperm sex determination, fluorescent *in situ* hybridization (FISH) using one, or two, sex-specific probe(s) is often the preferred method with successful hybridization signals often near to 100% (Rens *et al.*, 2001; Habermann *et al.*, 2005; Yan *et al.*, 2006). As an alternative to FISH, the use of PCR for single sperm typing is attractive due to the inherent simplicity and functionality of the method. Single sperm sex typing using a multiplex nested PCR to the testis determining gene (SRY) located only on the Y chromosome has been described in human sperm (Cui, 1997). The co-amplification of a control gene (ZP3) in this study was used to differentiate PCR failure from the absence of band due to X sperm. However, single template multiplex nested PCR can be difficult to optimize to ensure balanced amplification between the control PCR products and the sex-specific PCR products.

The amelogenin gene is advantageous for sex-testing as the reaction contains a built-in reaction control which detects PCR failure, therefore, does not require a control gene.

Amelogenin is an extracellular matrix protein secreted by ameloblasts during the development of tooth enamel (Sasaki and Shimokawa, 1995). Gibson *et al.* were first to describe the structure of the amelogenin gene in cattle as having two transcripts, one on each sex chromosome, of which the Y-transcript contained a 63 bp deletion (Gibson *et*

al., 1991). A method for determining the sex of bovine embryos was subsequently described amplifying the amelogenin loci by PCR (Ennis and Gallagher, 1994). Recently, Weikard *et al.* confirmed the 63 bp deletion, and overall sequence conservation, in nine members of the *Bovidae* family (Weikard *et al.*, 2006). Amelogenin has been successfully used to evaluate semen sex-ratio in pooled bovine DNA samples using capillary electrophoresis (Checa *et al.*, 2002). The objective of this study was to develop and validate a method for single sperm isolation and sex typing of single bull sperm using the amelogenin gene.

3.2. Materials and Methods

3.2.1. Sperm preparation

Sex sorted semen and unsorted semen was obtained from commercial distributors. Sperm were centrifuged at 800 x g for 5 minutes and resuspended in 1 x PBS supplemented with 5 mM glucose prior to use.

3.2.2. Manual isolation

Single sperm were individually isolated from poly-l-lysine coated standard glass microscope slides, containing between 100 and 200 sperm per slide. The poly-l-lysine can be substituted for the hydrophobic Sigmacote solution (Sigma, St. Louis, MO, USA) which improves recovery of sperm. Slides are suitable for use with standard procedures, e.g. immunolocalization and imaging. Selected single sperm were dissolved directly on the slide using 1 μ L of lysis buffer (200 mM KOH, 50 mM DTT) (Lien *et al.*, 1993) for 2 min at room temperature before transferring lysis solution to the PCR tube. Lysis procedure is checked microscopically and repeated as necessary to ensure complete recovery.

3.2.3. Isolation by flow-sorter

For single sperm sorting by flow cytometer, 2 million sperm were stained with 2 μ g/mL Hoechst 33342 fluorescent dye for 20 minutes (Invitrogen, Carlsbad, CA, USA).

Commercial sex-sorted sperm was already stained from sex-sorting, and therefore could be used directly for single sperm sorting. Single sperm were flow-sorted directly into 96-

well PCR plates containing 2 μ L of sperm lysis buffer (200 mM KOH, 50 mM DTT) (Lien *et al.*, 1993) using a FACS Aria flow-cytometer equipped with an automated cell deposition unit (ACDU) (BD Bioscience, San Jose, CA, USA). Single sperm were also collected into clear-bottom 96-well culture plates and viewed using an inverted microscope to record the error rate of the single sperm sorting procedure. For each plate 8-wells received 10 sperm, and 8-wells received no sperm, for positive and negative controls, respectively.

3.2.4. Primer design

The bovine amelogenin cDNA sequences (AMELX, GenBank accession no: NM_001014984; AMELY, GenBank accession no: NM_174240) were aligned using the ClustalW program (Chenna *et al.*, 2003) to identify the 63 bp deletion to which flanking nested PCR primers were designed. The outer nested primers (amelF1: 5'-CATGGTGCCAGCTCAGCAG-3' and amelR1: 5'-CCGCTTGGTCTTGTCTGTTGC-3') produced 367 bp and 304 bp X- and Y-amplicons, respectively. The inner nested primers (amelF2: 5'-CAGCAACCAATGATGCCAGTTC-3' and amelR2: 5'-GTCTTGTCTGTTGCTGGCCA-3') produced 329 bp and 266 bp X- and Y-amplicons, respectively. Primer specificity was verified using genomic DNA (5 ng/reaction) extracted from blood of male and female animals using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA).

3.2.5. PCR conditions

Lysis solution was incubated at 65 °C for 10 minutes prior to amplification to dissolve cell components. Neutralizing the lysis buffer as done in previous reports was deemed to be unnecessary if lysis component did not exceed 10% of total reaction volume. Nested PCR was carried out in 25 µL reactions containing 2.5 µL of 1X ThermoPol reaction buffer (New England BioLabs, Ipswich, MO, USA), 1.25 units of *Taq* polymerase, 0.4 µM of each primer, 200 µM of dNTP mix, 5% v/v glycerol. Both rounds of nested amplification were carried out in a Biometra TGradient thermocycler with one cycle of 95 °C for 4 min, 25 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, and a final extension step of 72 °C for 5 min, with 2 µL of the primary reaction used as template for the nested PCR. A 5 °C / s ramp speed was used in the outer primary PCR to reduce the total reaction time the *Taq* polymerase is exposed to the elevated pH caused by the lysis buffer. Reactions were separated by 3% agarose gel electrophoresis and analyzed using Typhoon Trio system with ImageQuant TL v2005 software (Amersham Biosciences, Piscataway, NJ, USA).

3.2.6. Statistics

A Chi square test was used to determine if the measured X:Y semen ratio from single sperm typing was significantly different from the expected ratio.

3.3. Results

3.3.1. Amelogenin amplification

The bovine amelogenin cDNA sequences were aligned using the ClustalW program (Chenna *et al.*, 2003), and conserved sequences in multiple cDNA were used to design nested PCR primers flanking the 63 bp deletion. To confirm the amplification of the amelogenin gene, genomic DNA was isolated from blood of Holstein male and female animals and used for PCR. As expected the bull produced both the 329 bp X-amplicon and the 266 bp Y-amplicon, while the cow produced only the 329 bp X-amplicon (Figure 10).

Figure 10. Amelogenin PCR product from 5 ng genomic DNA of bull (B) and cow (C). Male genomic DNA produces a 329 bp X-amplicon and a 266 bp Y-amplicon. Female genomic DNA produces only the 329 bp X-amplicon. Low range ladder (L) indicates amplicon size (bp).

3.3.2. Single sperm sex-typing

To test the accuracy of the amelogenin sex-typing method pooled unsorted sperm and sex-sorted sperm were evaluated using two different single sperm isolation methods.

Using the manual isolation method 125 single sperm were manually isolated, and lysed, on a microscope slide for single sperm sex-typing. In total, 120 single sperm were successfully typed using amelogenin nested PCR (Figure 11). The unsorted semen sample was found to contain 55.8% and 44.2% of X- and Y-sperm, respectively, which does not deviate significantly from the expected 50:50 ratio (Table 5). For the X-sorted sample, 91.2% of the sperm were found to be X-sperm, using the amelogenin single sperm testing method, which was not significantly different from the expected level determined by flow re-analysis

Figure 11. Single sperm sex-typing by nested amelogenin PCR. Six single sperm were manually isolated and sexed using amelogenin primers. Sperm 2, 5, were found to be X-sperm producing a 329 bp band. Sperm 1, 3, 4, 6 were found to be Y-sperm producing a 266 bp band. Also shown is 5 ng of male genomic DNA as a positive control (+) and a no-template negative control (-). Low range ladder (L) indicates amplicon size (bp).

Figure 12. Single sperm sex-typing by nested amelogenin PCR in X-sorted semen (**A**) and unsorted semen (**B**). X-sperm produce a 329 bp band and Y-sperm produce a 266 bp band. Also shown is 5 ng of male genomic DNA as a positive control (+) and a no-template negative control (-). Low range ladder (L) indicates amplicon size (bp).

While we demonstrated that single sperm could be manually isolated, in some cases a larger number of sperm need to be analyzed. Sex sorted sperm can be easily re-sorted individually into 96-well PCR plates using a flow-cytometer as the Hoechst dye used for sex-sorting remains in the sperm. Unsorted semen samples can also be easily stained with a fluorescent dye prior to sorting single sperm. The error rate in single sperm sorting, evaluated for each sample by sorting concurrently into clear-bottom 96-well plates, was on average 27.5% and 3.75% for wells containing no sperm, or multiple sperm, respectively. For X and Y sex sorted sperm, the flow re-analysis determined the samples to contain 92% and 95% X- and Y- sperm, respectively. For the X-sorted sample, 91.4% of the sperm were found to be X-sperm, using the amelogenin single sperm testing method, which was not significantly different from the expected level determined by flow re-analysis (Table 5). Likewise, for the Y-sorted sample, 94.0% of the sperm were found to be Y-sperm which was also not significantly different from the expected level (Table 5). The unsorted semen sample was found to contain 44.6% and 55.4% X- and Y-sperm, respectively, which does not deviate significantly from the expected 50:50 ratio in unsorted semen (Table 5).

Table 5. Measured X:Y ratio as determined by amelogenin single sperm sex-typing as compared the expected values by flow re-analysis for sorted sperm and the expected 50:50 ratio for unsorted pooled sperm. Fisher's exact test was used to compare the observed X:Y ratio to the expected ratio.

Semen Sample	Isolation method	Expected ratio		Amelogenin semen ratio determination				
		X-sperm (%)	Y-sperm (%)	n.	X-sperm (%)	Y-sperm (%)	P-value	fail rate (%)
X-sperm	manual	>90 ^a		68	91.2		0.74	1.4
unsorted	manual	50 ^b	50	52	55.8	44.2	0.87	5.5
X-sperm	flow-sort	92 ^a		185	91.4		0.99	22.9
Y-sperm	flow-sort		95 ^a	184		94.0	1.00	23.3
unsorted	flow-sort	50 ^b	50	56	44.6	55.4	0.91	30.0
Total n. sperm				545				

^a. Ratio was determined by commercial flow re-analysis for sorted sperm

^b. Ratio expected in unsorted semen: 50% X : 50% Y.

n. Number of sex typed sperms

3.4. Discussion

At initial stages of method development of novel sperm sexing techniques, the number of sexed sperm is often very low and traditional evaluation of sorting efficiency using insemination, flow cytometry, or quantitative PCR, is often inapplicable. In light of this, a single sperm sex-typing method that is reliable and easy to perform was developed for this project.

Several methods have been proposed to isolate single sperm for use in single cell typing including: micromanipulation (Iida *et al.*, 1996), low-melting agarose thin gel (Lien *et al.*, 1993), laser microdissection (Di Martino *et al.*, 2004), and single cell flow sorting (Shi *et al.*, 2001). Here we have developed a new simpler method for manual single sperm isolation that has the advantage of allowing the sperm to be mounted on a microscope slide and used for typical techniques such as immunolocalization or live/dead analysis prior to isolation and sex-typing. Using this method, we were able to sex-type single sperm at a 96% success rate (n=125) which was higher than reported success isolating by micromanipulation (Goradia *et al.*, 1991), and similar to success using agarose thin-gel isolation (Lien *et al.*, 1993). The failed samples (4%) were most likely due to loss of sperm during isolation, failure of sperm lysis, or failure in PCR reaction. In particular, the lysed sperm sticking to the slide was occasionally observed therefore extra care to re-check recovery of sperm was often necessary. There was no incidence of double bands indicative of multiple sperm or sperm contamination by free floating DNA present in semen. The manual isolation method could also be easily applied after a FISH

experiment to confirm the results directly on the same slides, and same sperm, used for hybridization. We also demonstrated that high throughput sorting of single sperm can be achieved using a flow-cytometer. The number of wells with no sperm was relatively high due to using the standard flow-nozzle not designed for sorting sperm, however, this number would be expected to be much lower with nozzle specially designed to orientate sperm properly (Johnson *et al.*, 1999; Garner, 2006).

Several different genes have been suggested for determining the sex of bovine embryos or sperm. Initially, single-sperm amplification was tested using a regular 50-cycle PCR amplification protocol using primer sets to the Y-linked testis determining gene (SRY), the X-linked proteolipid protein gene (PLP) (Parati *et al.*, 2006), as well as the amelogenin gene (AMEL). Only the PLP primer set was adequately sensitive (not shown), however, it did not function consistently in a multiplex amplification reaction, as would be needed for amplification control in single sperm typing. Nested PCR primers to the amelogenin deletion were therefore developed.

A typical PCR reaction uses ~5 ng of template, however, Weikard *et al.* found the lower limit for detection of amelogenin to be 40 pg of bovine gDNA (Weikard *et al.*, 2006). A single sperm contains approximately 3.2 pg of template (Pogany *et al.*, 1981), and therefore could not be reliably amplified without a nested amplification reaction. There are a number of advantages with using amelogenin PCR for single sperm sexing over other possible candidates. The nature of the amelogenin amplification does not rely on the lack of amplification to infer sex of the sperm as in other methods, as either X- or Y-

specific PCR fragment should be produced in every reaction. It also provides a built-in amplification control and therefore does not need to be multiplexed with an autosomal control gene. With any single template amplification occasional cross contamination is inevitable. To avoid contamination a laminar hood, dedicated pipettes for master mix preparation and the appropriate negative controls should be used. While the use of a separate contamination control is essential to identify instances of cross contamination, with amelogenin PCR there is additional control in each reaction. For example, contamination by the smaller Y-chromosome amelogenin PCR product in an X-sperm reaction will be easily detected as appearance of two bands on the gel. However, if the contaminating band is of the same size, for example, contamination by the X-chromosome amelogenin PCR product in an X-sperm reaction, the contamination will not be detected.

The amelogenin single sperm sex-typing method can also be extended for semen sex ratio determination by sex-typing many sperm. In all samples the calculated X:Y ratio was not significantly different from the results of the flow re-analysis data for sex sorted sperm or the predicted 50:50 expected ratios for unsorted sperm confirming the accuracy of the method. This indicates that even with relatively small evaluations (100 to 200 single sperm) accurate predictions of semen ratio can be achieved. Routine semen X:Y ratio determination using this method might be impractical given the large sample sizes required to generate high confidence predictions. In situations where sperm number is low, or sperm are very valuable, the smaller number of sperm required for semen ratio determination using this method compared to other methods would be beneficial. The

amelogenin single sperm sexing method would also be valuable in quality control of sex sorting and would integrate easily into conventional flow-sorting procedures as these flow-sorters are capable of direct single sperm sorting into PCR-plates.

3.5. Conclusion

We have found the amelogenin single sperm sexing method to be an effective tool in sex typing single sperm. It could be useful in evaluating new sperm-sexing methods, in investigating the roles of the sex-chromosomes in various sperm processes, and in diagnosing the origin of meiotic non-disjunction events. Furthermore, given the sensitivity of the nested PCR, this method could easily be adapted to sexing of single embryo blastomeres, cultured bovine cell lines, or in meat testing. The method is also relatively cost effective as the reaction can be performed for less than 25 cents per single sperm with the primary cost being *Taq* polymerase, which we have optimized to the lowest possible concentration. Although, the majority of our work was performed with the Holstein breed we have also successfully tested the amelogenin system using single sperm from Finncattle breed, a dual purpose breed of Landrace origin from Finland (data not shown). The inherent simplicity, reliability, built-in amplification and contamination control, and wide adaptability to various other techniques, make the amelogenin single sperm sexing method a valuable tool for single sperm analysis. With sex-sorted semen becoming more common in animal production, robust methods such as this will be beneficial in evaluating current flow-sorting methods and likely help to develop future novel sorting methods.

CHAPTER 4: MONOCLONAL APTAMER TESTING

4.1. Introduction

Aptamer pool testing, even with a selected pool, is confounded by the presence of multiple aptamers within the pool. Although selected aptamers have common features, slight differences in sequence will yield a variety of binding characteristics leading to apparent signal background during testing. Furthermore, pool composition is always variable after asymmetric PCR amplification making technical replication with pools difficult. Monoclonal testing, where single aptamer species are tested individually, allows for much better characterization of aptamers.

Monoclonal aptamer testing is standard procedure following most SELEX-type experiments to identify the most optimal aptamer. For example, monoclonal testing of twenty aptamer clones was used to identify candidate aptamers that specifically recognize leukemia cells mixed with normal bone marrow cells (Shangguan *et al.*, 2006). In another study that used the whole-cell selection approach to develop aptamers to proteins found in the tumor matrix, a single aptamer from 163 clones was selected for monoclonal testing based on motif characterization (Daniels *et al.*, 2003).

Monoclonal aptamers can be amplified from plasmid clones containing the aptamer sequence, or synthesized directly using automated oligonucleotide synthesis technology. The main advantage of direct synthesis is the absence of FITC-labeled primers as well as

PCR reaction components that complicate testing. Testing using synthetic oligonucleotides removes any need to normalize or correct data due to background fluorescence and removes aptamer variability during testing, allowing for technical replication.

For the ultimate goal of using aptamers for sperm sexing, a single X-aptamer, and/or a single Y-aptamer, needs to be identified. The SELEX procedure selected 17 X- and 17 Y-aptamers from a pool of 1.2×10^{14} random sequences, and subsequent *in silico* analyses identified three X-aptamers (#11, 12 and, 20), and one Y-aptamer (#24), as good candidates for further testing (chapter 2). The current objective is use the monoclonal approach to determine if one or more of these aptamers bind 1) specifically to sperm features; 2) specifically to X- or Y-sperm. Any aptamer meeting the criteria, would be subjected to determination of the sex-specific target, and optimal conditions for sperm sexing.

4.2. Materials and Methods

4.2.1. Aptamer preparation

Four aptamers (X-aptamer #11, #12, #20; Y-aptamer #24) were synthesized full-length (including original primer sequences) with FITC-fluorochrome at 5'-end (Sigma, St. Louis, MO, USA), as detailed in section 2.2.2. Synthesized aptamers were dissolved in TE buffer (10 mM Tris-HCl, pH 8.5, 1 mM EDTA) to a concentration of 100 μ M and stored protected from light at -20 °C. Unlabelled initial aptamer library (Sigma, St. Louis, MO, USA) served as competitor DNA during monoclonal testing. Aptamers (50 pmol), and if applicable competitor DNA (400 pmol), were separately diluted in 1X ThermoPol PCR reaction buffer (New England BioLabs, Ipswich, MO, USA) to a final volume of 20 μ L and allowed to fold by denaturation at 95 °C for 5 min followed by 72 °C for 5 min using thermocycler (Biometra TGradient) to mimic conditions of the last step of the aptamer amplification reaction used during selection (section 2.2.2). Folded aptamers were kept protected from light on ice until use (max time on ice: 60 min).

4.2.2. Sperm preparation

Cryopreserved unsorted sperm that were diluted and pooled when fresh (5 Holstein bulls), and cryopreserved X- and Y-sperm that were sex-sorted when fresh by flow cytometry (single Holstein bull), were used for monoclonal aptamer testing. Purity of sex-sorted sperm was determined by flow-resort analysis at the time of sorting to be 92% for X-sperm sample, and 95% for Y-sperm sample. Straws were thawed (37 °C, 5 min, sperm in commercial cryopreservation buffer), transferred to microcentrifuge tubes, and

allowed to cool to room temperature prior to use. Sperm were used directly (without washing) to minimize membrane damage.

4.2.3. Monoclonal aptamer testing with unsorted sperm

Aptamers-sperm incubations were carried out at room temperature in light protected polypropylene microcentrifuge tubes. The four aptamers (X-aptamer #11, #12, #20; Y-aptamer #24) were tested with competitor DNA blocking step with unsorted sperm. X-aptamer #20 was further tested without competitor DNA blocking step with unsorted sperm. Competitor DNA blocking step, if used, was performed as a separate incubation before aptamer-sperm incubation. Briefly, blocking step was performed by incubating 20 μL of prepared competitor solution, with 20 μL of prepared sperm (1.6×10^5 sperm), and 30 μL of selection buffer [1 x PBS (HyClone, Logan, UT, USA) supplemented with 5 mM glucose] for 15 min at room temperature. Sperm were then diluted to 400 μL , washed by centrifugation (400 x g, 7 min, room temperature), and resuspended in 20 μL of selection buffer. Aptamer-sperm incubation was performed by incubating 20 μL of prepared aptamers, with 20 μL of competitor DNA blocked sperm [or 20 μL of prepared unsorted sperm (1.6×10^5 sperm) if competitor step was not performed], and 30 μL of selection buffer, for 10 min at room temperature. Hoechst 33342 DNA fluorescent stain (Invitrogen, Carlsbad, CA, USA) was then added (2 $\mu\text{g/mL}$; final concentration) and incubated for an additional 5 min at room temperature. After aptamer incubation, sperm were diluted to 400 μL , washed by centrifugation (400 x g, 7 min, room temperature), and the pellet resuspended in 50 μL of selection buffer. Sperm were then fixed at a 1:1

ratio (vol:vol; final volume 100 μ l) with 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for 10 min.

4.2.4. Monoclonal X-aptamer #20 testing with flow-cytometry sex-sorted sperm

X-aptamer #20 was tested with competitor DNA for sex-sorted sperm. Competitor-sperm, and aptamer-sperm, incubations were performed as described above using 1.6×10^5 sex-sorted sperm, which already contained nuclear stain from sex-sorting process and therefore no Hoechst 33342 dye was added.

4.2.5. Fluorescence microscopy

Standard glass microscope slides were washed in 100% ethanol for 60 min, rinsed in deionized water, and allowed to fully dry. Fixed sperm (20 μ L) were smeared onto prepared slides, allowed to fully dry, and were mounted using Prolong antifade mounting medium (Invitrogen, Carlsbad, CA, USA). Slides were allowed to cure overnight before microscopy. Sperm were viewed using fluorescence light microscope (DMR, Leica Microsystems, Wetzlar, Germany; fitted with 100W fluorescent lamp; FITC excitation filter: 450-490nm; Hoechst 33342 excitation filter: 360nm) and captured using OpenLab (version 4.04) software (Improvision, Coventry, England).

4.2.6. Single sperm sexing

Individual sperm were sex-typed using the amelogenin single sperm sexing method described in chapter 3. Standard glass microscope slides were cleaned as described above, then coated with Sigmacote solution according to recommended procedures

(Sigma, St. Louis, MO, USA), and blocked overnight (10 mM Tris, 0.05% v/v tween 20, 150 mM NaCl, 5% milk). Unsorted sperm were prepared and incubated with aptamers as described above for unsorted sperm with competitor DNA. After sperm fixing, samples were diluted 1:5 (vol:vol) with selection buffer. Diluted sperm (40 μ L) were smeared onto prepared slides, allowed to fully dry, and were mounted using Prolong antifade mounting medium (Invitrogen, Carlsbad, CA, USA). Well-isolated individual sperm were imaged as described above and location(s) marked with permanent marker. After imaging, coverslips were removed, and residual mounting medium was removed by soaking slide in 100% ethanol until fully dissolved. Slides were allowed to dry and single sperm were recovered and typed for amelogenin as described in chapter 3 (section 3.2).

4.2.7. Data analysis

For testing of X-aptamer #20 with unsorted sperm, 10 images were captured and all sperm were classified as positive, or negative, for binding of X-aptamer #20 (63 total sperm counted). For testing of X-aptamer #20 with both X- and Y-sorted sperm, 200 sperm were viewed on the microscope and classified for asymmetrical binding. No statistical data analysis was performed on counting for either type of sperm.

4.3. Results

The four candidate aptamers (X-aptamer #11, #12, #20, Y-aptamer #24) were tested by fluorescence microscopy to identify conserved aptamer binding patterns indicative of specific aptamer binding (Figure 13). X-aptamer #12 did not have detectable aptamer fluorescence by microscopy (Figure 13B). X-aptamer #11 and Y-aptamer #24 did have sporadic aptamer binding over sperm acrosome but no distinct pattern was found (Figure 13A,D). X-aptamer #20 fluorescence was observed over sperm head and midpiece with occasional spots of increased fluorescence on acrosomal ridge and tail. In addition to this fluorescence, a sub-population of sperm were observed to display a surprising asymmetrical binding pattern with a single bright spot in a highly conserved location on the lateral edge of acrosome (Figure 13C).

X-aptamer #20 was further tested with pooled cryopreserved unsorted semen from five bulls to confirm the asymmetrical binding pattern was not the result of a bull specific feature. Approximately half of the pooled sperm (28/63 sperm) displayed the characteristic asymmetrical binding pattern that would be expected if binding were sex-specific (Figure 14).

Figure 13. Monoclonal aptamer testing. Unsorted frozen/thaw bull sperm were incubated with FITC-labeled aptamers selected for sex specificity and viewed by fluorescence (left column) and phase (right column) microscopy. X-aptamer #11 (A), X-aptamer #12 (B), X-aptamer #20 (C), and Y-aptamer #24 (D), (scale bar: 10um)

Figure 14. Observed binding pattern of X-aptamer #20 when incubated with pooled unsorted bull sperm counterstained with Hoechst 33342. Arrow indicates location of asymmetrical aptamer binding. (scale bar: 15 μ m)

Incubating fluorescent X-aptamer #20 with the same X/Y-sorted sperm used for selection found that the asymmetrical pattern was present on a greater percentage of X-sorted sperm than Y-sorted sperm (60/200 X-sperm; 15/200 Y-sperm; Figure 15). Some variability in the intensity of the asymmetrical fluorescence binding was observed; in particular, some X-sperm displayed very bright asymmetrical spot, while other sperm had noticeably less intense fluorescent spot.

Unsorted sperm incubated with X-aptamer #20 in the absence of competitor DNA to block non-specific binding showed no fluorescence over the sperm head, but a dimmer asymmetrical spot was still found in a similar sub-population of sperm, in the same highly conserved location (Figure 16).

When the sex-specificity of X-aptamer #20's binding was further tested on 11 randomly-selected sperm from unsorted semen, three displayed the asymmetrical pattern and eight did not (Figure 17). Single sperm amelogenin sex typing determined that all three sperm displaying the asymmetrical pattern were X-sperm, while of the eight single sperm that did not demonstrate the asymmetrical patterns 6 were found to be Y-sperm while 2 were found to be X-sperm.

Figure 15. Typical sperm micrographs after exposure to FITC-labeled X-aptamer #20. X-sorted sperm (A) and Y-sorted sperm (B). (scale bar: 15 μm)

Figure 16. Effect of competitor DNA presence (A), and absence (B), on the binding pattern of X-aptamer #20 by fluorescence microscopy. Arrow indicates location of asymmetric aptamer binding. (scale bar: 10um)

Figure 17. Amelogenin single sperm sexing of unsorted pooled sperm incubated with X-aptamer #20. Sperm sex-type shown as X or Y as determined by Amelogenin single sperm sexing. Arrowhead indicates location of asymmetrical aptamer binding. (red scale bar: 15 μ m; white scale bar: 10 μ m)

4.4. Discussion

Four candidate aptamers (X-aptamer #11, #12, #20, and Y-aptamer #24) were examined for conserved aptamer binding patterns, representative of aptamer binding to a specific target, using a monoclonal approach. Of the four aptamers, one aptamer in particular, X-aptamer #20, appears to have specific binding and potential for identifying sperm bearing X-chromosomes.

X-aptamer #12 was chosen as a candidate for further testing because it was found to be the highest scoring of all sequenced X-aptamers by the X-PSASM and contains two copies of the consensus X-motif (sections: 2.3.3; 2.3.6). However, no detectable aptamer fluorescence was found on sperm by fluorescence microscopy. This would suggest that either X-aptamer #12 does not bind to sperm, or binds at such low amounts that it was not detectable by microscopy.

Y-aptamer #24 and X-aptamer #11 were selected as candidates based on high and low scoring by the Y-PSASM, respectively (section 2.3.3). Both X-aptamer #11 and Y-aptamer #24 were found to have aptamer binding sporadically over sperm acrosome in some sperm, however, binding appeared to be random with no consistent pattern. This would suggest that although these aptamers do bind to sperm, the binding was non-specific and likely unrelated to sex-type of sperm.

X-aptamer #20 was selected as a candidate for testing because it contains four statistically relevant copies of the consensus X-motif - three of which occur as tandem

repeats (section 2.3.6). Of the four aptamers selected for monoclonal aptamer testing only X-aptamer #20 displayed a conserved binding pattern by fluorescence microscopy. X-aptamer #20 binding creates a very bright concentrated spot of fluorescence on the lateral edge of sperm acrosome. While X-aptamer #20 produces a very obvious binding pattern, it is possible that the other aptamers do bind to sperm in a specific pattern/location but the fluorescence from individual aptamer(s) is low and cannot be detected visually by microscopy.

If X-aptamer #20 was selected to sex-specific features on X-sperm, differential aptamer binding patterns should be observed between X- and Y- sorted sperm. The asymmetrical binding was seen more often in X-sorted sperm than Y-sorted sperm. Thirty percent of X-sorted sperm displayed the asymmetrical pattern, compared to only 7.5% of Y-sorted sperm, suggesting a potential X-preference. However, if X-aptamer #20 is specific to X-sperm, then the aptamer would be expected to show the characteristic asymmetric binding pattern in 92% of the X-sorted sperm population (expect sort-purity to be ~92% X-sperm). X-aptamer #20 binding to a sub-population of target X-bearing sperm is similar to that reported previously in a similar experiment where certain candidate aptamers after whole-cell selection would bind only 20-40% of target cells. This was likely due to over-specification of aptamers to a particular molecular signature, cell-type, or cell-stage, within the target population (Shangguan *et al.*, 2006). Also, it has been shown that at least two populations of Y-sperm exist differing in membrane properties (Cartwright *et al.*, 1993); therefore it is possible that aptamers only interact with a sub-population of sex-sorted sperm. The Y-sorted sample used here for testing, was

contaminated with approximately 5-10% X-sperm, therefore the asymmetrical binding to Y-sorted sperm could be at least in part due to X-sperm present in the sample.

Binding of aptamer to only a sub-population of target sperm might also be due to removal of sex specific markers during flow sorting by chemical stresses, high dilution, high pressure, and significant mechanical forces (Parrilla *et al.*, 2004; Spinaci *et al.*, 2005).

Such damage would explain the differences in the ratio of sorted and unsorted sperm that asymmetrically bound X-aptamer #20. X-aptamer #20 bound only 30% of sex sorted X-sperm (expected 92% of sperm were X sperm), but bound 44% of unsorted frozen-thaw sperm (expected ~50% of sperm were X-sperm). Assuming the binding was sex-specific, in the sorted sample X-aptamer #20 was able to bind 33% (30/92) of available X-sperm; while in unsorted sperm X-aptamer #20 was able to bind 88% (44/50) of available X-sperm. This result also would suggest that the aptamers were targeting a feature relevant to regular unsorted sperm and were not targeting a feature, or defect, present only in sex-sorted sperm. This was considered a major obstacle of this research, as the sorted sperm used for selection were considerably different, particularly in membrane structure, due to damage inflicted by the sorting process. Confirmation in a larger study that all sperm with asymmetric staining are X-sperm, coupled with this conserved binding to regular sperm, will promote use of X-aptamer #20 in novel sperm sorting techniques.

In addition to the asymmetrical spot of fluorescence, X-aptamer #20 also produces sporadic fluorescence over sperm head in the presence of competitor DNA. This additional fluorescence, which was thought to be non-specific, was only found in the

presence of competitor, suggesting that the competitor creates the non-specific binding of X-aptamer #20. Likely, the competitor first bound randomly to sperm surface, then through an aptamer-DNA to competitor-DNA interaction the aptamers were bridged to the sperm surface creating the observed sporadic pattern. This unexpected role of competitor DNA would have hampered selection by allowing non-specific aptamers to remain in the selected aptamer pool. This might explain why X-aptamer #11 and Y-aptamer #24 were not found to have specific binding pattern by monoclonal testing.

Foreign DNA-binding to sperm has been well documented in several species.

Mammalian sperm contain a conserved family of sperm membrane DNA binding proteins that are thought to be involved in exogenous DNA binding and internalization (Zani *et al.*, 1995). It is assumed that the specific binding of X-aptamer #20 is not to these proteins, as they are located in the equatorial segment and sub-acrosomal region of sperm, not in the apical acrosome region where X-aptamer #20's asymmetrical binding occurs (Atkinson *et al.*, 1991; Spadafora, 1998). However, the additional sporadic binding also seen with X-aptamer #20 when competitor DNA was used, was quite likely mediated, at least to some extent, by these foreign DNA-binding proteins. Reports of internalized foreign DNA show transport/localization to the sub-acrosomal region, therefore, if X-aptamer #20 was being internalized by a DNA-uptake mechanism, aptamer fluorescence should be found in the sub-acrosomal region - not on lateral edge of acrosome (Anzar and Buhr, 2006). The location of X-aptamer #20 binding was very conserved suggesting a distinct target present in that location. When sperm were counterstained with Hoechst 33342 DNA binding stain, the asymmetrical aptamer

binding appears to be exterior to the nuclear envelope. This does suggest that X-aptamer #20 was not binding to complementary genomic DNA motifs. Fluorescence light-microscopy seems to show that the location of binding was on the exterior face of the plasma membrane; however, this type of microscopy cannot definitively distinguish between the extracellular and cytoplasmic surfaces of the plasma membrane. Foreign DNA binding to the outer acrosomal membrane has been observed previously in a very small subpopulation of bull sperm (<1%), possibly acrosome reacted sperm, in a similar region to X-aptamer #20 binding, but more broadly distributed over the entire anterior acrosome (Atkinson *et al.*, 1991), rather than in the discrete asymmetrical pattern displayed by X-aptamer#20. Cryopreserved sex-sorted sperm are known to prematurely acrosome-react (Moce *et al.*, 2006), therefore it is conceivable that the subpopulation of sperm that bind aptamers asymmetrically are in fact acrosome reacted. More research will need to be conducted to determine if the subpopulation in question is in some way different, for example: capacitated, acrosome reacted, hyperactivated, or membrane damaged.

Interestingly, the fluorescence micrographs suggest that at least X-sperm might have right and left sides with non-identical features. Sperm asymmetry is found in nature; for example, murine rodents possess a highly asymmetrical sperm morphology including an apical hook and an asymmetrical acrosome (Breed, 2004). It has been also reported that sperm in pig, cow, sheep, horse, and rabbit have dorsal-ventral asymmetry with one surface of the head showing slight convexity and the other is either flat or slightly

concave (Dziuk and Dickmann, 1965; Green, 1988). So it might not be surprising that sperm in addition to dorsal-ventral asymmetry can also show left-right asymmetry.

The asymmetrical binding of X-aptamer #20 produces a very intense fluorescence signal. If this binding was confirmed to be sex-specific this would be efficient for sorting sperm using flow-cytometry or other fluorescent-dependent strategies. It is however, difficult to suggest that this signal is emitted by a single aptamer and therefore single fluorescein molecule. Either many aptamers were co-localizing to this specific target producing a strong signal (aptamer aggregation) or many targets were present in this region for multiple aptamers to bind (target aggregation). With the definitive, and numerous, previous failures to identify any sex-specific marker using antibodies, or other methods, it seems unlikely that many available targets could exist.

One possibility is that while there is only single target site, multiple aptamers can interact with each other leading to signal amplification. The aptamer design does contain a 6-nucleotide complementary sequence (5'- TCCATCTCTTCTGTATGTCGAGATCTA-(N)₃₆- TAGATCTCCTAACCGACTCCGTTAATT-3') in the primer region that could mediate concatemerization of aptamers into a chain. This aptamer-aptamer interaction might stabilize the functional binding site of the lead-aptamer and produce much stronger fluorescent signal. Pre-assembly of oligonucleotide concatemers has been shown to considerably increase the ability to bind whole cells as compared to the same free oligonucleotides (Simonova *et al.*, 2006). We have observed that the asymmetric binding pattern was variable and brighter in the presence of competitor DNA. This could indicate

that competitor DNA was also acting to bridge multiple aptamers together producing a stronger fluorescence signal.

X-aptamer #20 was found to be G-rich in the aptamer region with multiple G-repeats. Such G richness might indicate higher structural stability of GC rich aptamers, and such G-rich aptamers can be preferred during whole-cell selection (Wu *et al.*, 2003; Wu *et al.*, 2004). G-rich DNA can form inter- and intra-molecular four-stranded structures, referred to as G-quartets (Gilbert and Feigon, 1999; Burge *et al.*, 2006) that contain four coplanar guanines stabilized by Hoogsteen type base pairing. An array of G-quartets stacked on top of each other produces tetrad-helical G-quadruplexes that are very stable and play important biological roles, for example, as telomeric sequences protecting the end of chromosomes. Interestingly, previous selection of DNA aptamers chose G-quadruplexes (Allen *et al.*, 1995). Therefore it is possible that X aptamer #20 could form such G-quartets, creating G-quadruplex aggregates either within the single aptamer, across multiple aptamers, or between aptamer and competitor DNA, as a stabilization mechanism. G-quartets are specifically stabilized by potassium (Mergny et al, 1998). We did observe that X-aptamer #20 binding was very dependent on the presence of magnesium and potassium ions (data not shown), originally arising from PCR buffer, which might be required for this aptamer-aptamer or aptamer-competitor interaction.

Although aptamer aggregation could produce the observed intensely fluorescent spot, target aggregation could occur if multiple targets from over sperm head at some point aggregate, thereby producing the observed strong signal. The aggregation of many

fluorescent aptamers into a single spot on sperm membrane was reminiscent of a patching and capping type mechanism. Patching and capping involves ligand-receptor binding causing association of receptor complexes (patching), followed by grouping of patches into large aggregates at one pole of cell (capping). Patching and capping of fluorescence antibody will appear as a concentrated spot of fluorescence on cell surface (Bourguignon, 1979). Antibody induced patching (Gaunt *et al.*, 1983) and capping (Trimmer and Vacquier, 1988), and shedding (Shaha, 1994) has been reported previously in sperm. In these studies, capping appeared as a fluorescent ring over entire anterior acrosome, however, this dissolved into a single spot during the shedding process (Shaha, 1994). Concentration of X-aptamer #20 targets by similar patching and capping mechanism would result in excessively bright fluorescence at a single spot, and since patching and capping are time and temperature dependent, the absence of an asymmetrical spot on certain X-sperm could be due to either unfinished capping or complete shedding of aptamer bound target. However, when fluorescent spots were produced due to patching and capping in sperm the location was not conserved as was seen for X-aptamer #20 (Nikolaeva *et al.*, 2000). The development of aptamers to cancer cells using whole-cell approach also produced intense fluorescence in conserved spots (Shangguan *et al.*, 2006); therefore this pattern of binding might be a common feature of aptamers selected to intact cell membranes.

Preliminary data using single sperm typing seems to confirm that sperm with asymmetrical binding were X-sperm while sperm with no asymmetrical pattern were most often Y-sperm. In the eleven samples sex-typed there have been no incidences of

false positive (X-aptamer asymmetrical pattern on Y-sperm), although false negatives have been observed (absence of asymmetrical pattern in two X-sperm). More sperm will need to be sex-typed to confirm this finding. If X-aptamer #20 does bind to a specific target in X-sperm this would be the first identification of a sex-specific surface marker in sperm.

4.5. Conclusion

Monoclonal aptamer testing of X-aptamer #20 revealed a conserved asymmetrical binding pattern in both unsorted and sex-sorted sperm. This asymmetrical binding pattern was more often seen with X-sorted, as compared to Y-sorted sperm, suggesting that aptamer-sperm interaction might be sex-specific. Evidence for sex-specificity was supported by fluorescence micrographs of unsorted sperm where X-aptamer #20 produces asymmetrical binding pattern with expected ~50% ratio of sperm and furthermore by single sperm sex-typing. The extracellular face of the head plasma membrane appears to be the site of asymmetrical aptamer binding. Larger-scale studies into the sex-specificity, and target of, X-aptamer #20 are necessary to confirm that binding is sex-specific.

CHAPTER 5: GENERAL DISCUSSION

5.1. Introduction

The ultimate goal of this research was to develop aptamer probes capable of resolving molecular differences between X- and Y- bull sperm. A procedure was developed for the selection of aptamers to live sperm and applied here to derive sex-specific aptamers to bovine sperm. Selected X- and Y-aptamer pools displayed increased binding to their respective X- and Y-sorted sperm. Characterization of selected aptamer sequences showed that the selected X- and Y-aptamer pools each had unique secondary structures, and unique sequence motifs. From the trillion aptamers initially screened, one particular aptamer, X-aptamer #20, was identified that binds to a specific target on sperm membrane and shows promise for sex-specific binding to X-sperm.

5.2. Suitability of aptamers to achieve research objectives

Aptamers are small molecular probes that are able bind to specific targets within a complex cellular environment, making them valuable tools for molecular recognition, biomarker discovery, and affinity purification. Recently, aptamer-based technologies have proven valuable in cancer research for differentiating cancerous cells from regular cells and for identifying key markers of abnormal cell types. Conceptually, developing aptamers to differentiate cancerous cells from normal cells is analogous to this project's goal of differentiating the molecular cell-surface signatures of X- and Y-sperm.

The lack of known sex-specific molecular marker(s) on sperm, makes aptamers the perfect tool for identifying molecular differences, because they can be applied on a

presumed difference with no preconceived bias as to the nature of a potential marker. Furthermore, the scope of potential biomarker types is infinite as aptamers concurrently screen all types of cellular molecules (in their native environment) as well as cellular shapes, and ionic or charge based features.

Aptamers are often compared to antibody-based methods as these two technologies have similar applications. Properly selected aptamers typically outperform antibodies in specificity, and affinity, for the respective target; and antibody-based approaches have been previously found not adequately sensitive to identify any molecular difference between X- and Y-sperm. No functional 'omics based technologies, the most common approach for wide-scope comparison of two cell types, have been applied yet to identifying molecular differences between X- and Y-sperm but are expected in the near future as these technologies have been found useful in sperm fertility research (Ostermeier *et al.*, 2002). However, uses of these technologies are limited to certain types of molecules (proteins, transcripts, etc) and are often applied under non-native conditions. Given the previous failures to identify any molecular difference between sperm it is possible that differences are within the same target molecule, for example different conformation of the same protein, and therefore would not be detected using 'omics approaches but may be detectable using aptamers. Furthermore, as 'omics approaches rely heavily on database information, if the target molecule has not been previously characterized it may not be easily identified. This may be particularly important in this scenario, as the Y-chromosome is not well characterized in proteomic and transcriptomic databases.

Despite the wide usage of aptamers in other areas, this research is, to our knowledge, the first to apply aptamers to the sperm field. A method for the selection of aptamers to sperm was developed, tested, and shown here to be suitable for the selection of aptamers to sperm features. The aptamer-selection methodology developed here would be useful in other sperm research for the identification of molecular difference between two sperm populations. For example, aptamers could be developed to characterize markers of live/dead sperm, sperm fertility, and capacitation or acrosomal status.

5.3. Sexing single sperm

In addition to developing methods for selection of aptamers to sperm, a simple, reliable method for determining the sex of single sperm was also developed. The amelogenin single sperm sexing method allows for direct sex-typing of individual sperm, for correlation of sperm-sex with other attributes detected by flow-cytometry or microscopy. For example, the Amelogenin method could be used to validate fluorescent *in situ* hybridization (FISH) results using the same slides and same sperm. In this study, the Amelogenin method was useful for relating aptamer-binding patterns to sex-chromosome identity of individual sperm. This method is expected to be useful to other researchers investigating the role of the sex-chromosomes in various processes, or in small-scale testing of new sperm-sexing methods.

5.4. Selecting aptamers specific to sperm sex chromosomes

Two separate aptamer selection procedures were performed to select both a X-aptamer and a Y-aptamer using flow-cytometry sex-sorted sperm as the target during whole cell selection. A candidate X-aptamer would be one that has the ability to bind to a specific target found on sperm carrying the X-chromosome (X-sperm) but not sperm carrying the Y-chromosome (Y-sperm). Likewise, a candidate Y-aptamer would be one that has the ability to bind to a specific target on Y-sperm but not X-sperm.

Candidate X- and Y-aptamers were selected using a modified version of the systematic evolution of ligands by exponential enrichment (SELEX) procedure to screen aptamers. The *in vitro* evolution of aptamer pools (SELEX) was performed for 20 cycles using negative selection (in rounds 3-20) and competitor DNA (in rounds 8-20) to favor selection of sex-specific X- and Y-aptamers. Measurable evolution of aptamer pools is typically detectable after 7-10 rounds of selection using SELEX procedure (Stoltenburg *et al.*, 2005; Shangguan *et al.*, 2006). To ensure aptamer evolution was proceeding as expected, and warranted continuing, the X- and Y-aptamer pools from selection rounds 7-11 were evaluated using flow-cytometry, fluorescent binding assays, and fluorescence microscopy (chapter 2). These results confirmed that selection was working and the X- and Y-aptamer pools were being selected to two unique features on sperm. Furthermore, the testing showed that the selected X- and Y-aptamer pools could bind more to X- and Y-sorted sperm, respectively, than the opposite sex of sperm, suggesting that aptamers were gaining the ability to differentiate X- and Y-sperm. Selection continued for 20-selection cycles, after which the X- and Y-aptamer pools were cloned and sequenced to

identify enriched sequences. From the 120 trillion aptamers initially screened using the SELEX procedure, 34-candidate aptamers (17 X-aptamers, 17 Y-aptamers) were identified by aptamer sequencing (Table 2). The candidate aptamers were analyzed using software based sequence analysis to infer the relatedness within X/Y-aptamer groups, predict the secondary structure of the aptamers (Figure 9), and identify conserved motifs unique to X- and Y-aptamer groups (Table 3,4).

Results from during aptamer selection, and testing of selected aptamers post selection, were on par with previous reports of selecting aptamers using similar methods. Flow-cytometry testing of selection round 7 aptamer pool binding to sex-sorted sperm found that the magnitude of curve shift (degree of selection) were similar to that of selection round 6 aptamer pools selected to cancerous human T-cell population (Shangguan *et al.*, 2006). This indicates that the selection of aptamers in this study occurred at a similar rate to previous report of selecting aptamers using negative-positive selection scheme to whole cells. Secondary structure prediction using *mfold* software found conserved stem-loop structures in consensus X-motifs within selected X-aptamers were similar to the stem-loop structures in aptamers selected to streptavidin beads, which suggests that the selected aptamers form characteristic aptamer shapes (Stoltenburg *et al.*, 2005).

Based on sequence analysis the 34-candidate aptamers were further narrowed to identify four aptamers (X-aptamer #11, #12, #20, Y-aptamer #24) for further testing.

Fluorescence microscopy subsequently found X-aptamer #20 to be the only one of the four aptamers with a visually-obvious specific binding pattern, confirming the selection

of a single specific aptamer to bull sperm from the initial pool. Binding of X-aptamer #20 was to approximately half of sperm in unsorted semen (Figure 13,14), and furthermore showed preference for X-sperm when tested with sex-sorted sperm (Figure 15). To further investigate the sex-specificity of X-aptamer #20, sperm with the asymmetric aptamer binding were confirmed to be X-sperm using the amelogenin single sperm sexing method (Figure 17). There were no false positives (sperm with asymmetric spot identified to be Y-sperm), and limited occurrence of false negatives. These results are strongly supportive of X-aptamer #20 being sex-specific for X-sperm, however, more testing is required. If absence of false positive detection continues, single sperm sexing of 100 sperm should be sufficient to provide evidence for sex-specificity of X-aptamer #20.

5.5. Impact of methodology used

In this study, aptamer selection was performed for a predetermined 20 selection cycles (chapter 2). Sequencing of selection round 20 pools found no aptamers with the exact same, or obviously similar sequence, suggesting that aptamer pools were still complex. Twenty selection cycles have been used in other whole-cell SELEX studies to successfully develop aptamers (Daniels *et al.*, 2003; Shangguan *et al.*, 2006). Selection should not continue indefinitely because as aptamer pools become selected the degree of selection for the desired trait slows and selection to other unknown traits is increased. In this, and other reports of whole-cell aptamer selection, even after 20 selection cycles the selected aptamer were over-specified to a sub-population of target cells (Shangguan *et al.*, 2006). This suggests that if more than 20 selection cycles are performed, the risk of developing aptamers that only function with certain sub-populations is increased.

The approach used here to analyze aptamer raw sequence data from more complex pools, using software based approach, was effective in identifying X-aptamer #20 as a candidate aptamer. Furthermore, identifying the important features of aptamers, such as conserved motifs and unique structure, is simpler with many selected aptamer sequences (more complex pool) as oppose to one specific aptamer. Undoubtedly, sequence data from more than 34 aptamers would be beneficial to fully characterize the selected pools and should still be performed in a larger-scale study. The aptamer library used in this study contains BglII restriction sites flanking the 36-nucleotide random region, which would allow aptamers to be concatemerized together during cloning yielding several aptamer sequences from a single sequencing reaction. Also, new sequencing technology can determine the sequence of more than 1,000,000 individual sequences per 10 hour run, which would allow characterization of essentially every aptamer in selected pool (454 Life Sciences, Branford, CT, USA).

Competitor DNA used here to block non-specific binding of aptamers to sperm during selection might have actually hindered the selection of specific aptamers. Fluorescence microscopy testing with X-aptamer #20 found that in addition to the asymmetric specific-target binding, in the presence of competitor DNA, X-aptamer #20 also binds randomly over entire acrosome. This apparent non-specific binding was not observed when X-aptamer #20 was incubated without competitor present (Figure 16). Therefore it seems that the competitor, while blocking non-specific sites, also bridges aptamers to sperm. During selection this would have resulted in aptamers being selected for without binding

to a specific target on sperm. Blocking non-specific binding is important during selection; however, another non DNA-based method might limit this unanticipated role of competitor DNA.

Aptamers in this study were selected to frozen-thaw sex-sorted sperm but found to remain functional in frozen-thaw non-sorted sperm as confirmed by testing of X-aptamer #20 with sorted and non-sorted sperm. This result would suggest that the aptamers were not targeting a feature, or defect, present only in sex-sorted sperm. This was considered a major obstacle of this research, as the sorted sperm used for selection were considerably different than normal sperm, particularly in membrane structure, due to damage inflicted by the sorting process. However, aptamers were selected under conditions favorable to selection, which may not be optimal conditions for sperm viability. For example, during aptamer selection, sperm were kept at room temperature for 90-120 min under non-protecting buffer conditions. It is unclear how aptamers selected under these conditions will function in conditions more conducive to sperm viability (ie: sperm in seminal plasma at ~37°C). Also aptamers have not yet been extensively tested with non-frozen sperm, therefore the applicability of these aptamers to fresh sperm is not known. Given proof of principal, in this study, for the selection of aptamers to sperm, if needed, it would be possible to re-select aptamers under different conditions more suitable to viable sperm.

5.6. Implications and future approaches

The identification of X-aptamer #20 as a specific probe to bull sperm, with what appears to be sex-specific binding, highlights the potential of aptamers as molecular probes and the power of the SELEX approach. If X-aptamer #20 sex-specificity were confirmed in a larger study, it would be the first identification of a sex-specific marker in any mammalian sperm. This would hold significant scientific implications to the sperm field, as the current model of spermatogenesis does not readily allow for the occurrence of a sex-marker.

This research lays the groundwork for several future larger-scale investigations. If X-aptamer #20 binding can be confirmed to be sex-specific, the target of aptamer binding would need to be resolved, and the mechanism leading to such a marker characterized. Direct application of aptamer(s) developed in this study, or the target identified by aptamers, for differentiation of X/Y-sperm might allow the development of novel sperm sexing methods. More efficient sperm sorting methods would decrease the current cost-premium on sorted sperm allowing wider adoption of sexed sperm. In particular, if marker is conserved to other livestock species, it would allow the development of new sperm-sexing technologies for species where current flow-cytometry methods are impractical.

Underlying, albeit perhaps somewhat peripheral to, the results of this research, are two questions: what is the function of a sperm sex-specific marker? And, how could such a marker have arisen, given the barriers in place to ensure sperm are phenotypically

equivalent? A logical correlate of a sex-specific marker in sperm is that sex-selection must occur as a natural process. It is possible that the sperm sex marker identified in this research, by X-aptamer #20, serves to label X-sperm for initiation of an endogenous sex-selection mechanism. The mechanism(s) allowing sperm to overcome barriers inhibiting X/Y-sperm differences leading to a sex-specific marker are not currently known. Sperm are thought to be biochemically and functionally equal despite being genetically different primarily due to free sharing of cell constituents across connecting intercellular bridges between developing sperm. However, sperm are not perfectly phenotypically equivalent. Different sub populations of sperm are quite common with relation to sperm morphology, motility, capacitation status, survival of cryopreservation, and fertilization ability (Rodriguez-Martinez, 2006). Even in our experiment it was clear that only ~40% of the sperm were able to bind the initial aptamers (Figure 6) which again indicates that sperm were non-identical and their phenotype and function might at least partially reflect underlying genotype. Furthermore, we sex-typed 51 single sperm produced from an infertile bull that produces drastically abnormal sperm diagnosed as a meiotic non-disjunction failure (data not shown). Sperm from this bull have noticeably varying phenotype suggesting that sharing was obviously not equal, nor complete, during sperm development. Therefore it is plausible that products of sex chromosome linked genes might also not be ubiquitously shared. Several smaller soluble proteins are shared between sperm; however, it is possible that a sex specific membrane protein, such as the membrane-target of X-aptamer #20, might be less readily shared. For example, the lack of sharing (LOS) hypothesis has been shown using Sperm Adhesion Molecule 1 (Spam 1), a sperm membrane protein which is not shared due to compartmentalization of the

transcript and anchoring to the cytoskeleton (Martin-DeLeon *et al.*, 2005). Therefore it is possible that the marker identified in this research by X-aptamer #20, exists as a result of the underlying X/Y genotype perhaps by escaping transcript sharing through a similar RNA compartmentalization mechanism. This type of mechanism could result in a sex-marker from a non-sex-linked gene product, which might explain why previous studies limited exclusively for sex-linked markers have ultimately failed.

5.7. Conclusion

A modified systematic evolution of ligands by exponential enrichment procedure was developed that successfully selected two families of aptamers to sorted X- and Y-bull sperm. The basic selection of aptamers was tested from multiple angles and, although the various methods used to test and characterize the aptamer binding are not necessarily definitive individually, together their results show that the two selected pools display unique, and deliberate, properties. The X- and Y-aptamer families have increased binding to X- and Y-sorted sperm, respectively, and contain conserved unique motifs. One aptamer, X-aptamer #20, identified a conserved asymmetrical target on sperm that, furthermore, appears specific to X-sperm. The sex-specificity and applicability of X-aptamer #20, or any other single aptamer, for bull sperm requires in-depth testing to confirm this very exciting and innovative finding, and fully realize its scientific, biological and commercial implications.

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