CRYOPRESERVATION OF BOVINE SEMEN IN EGG YOLK BASED EXTENDERS

A Thesis Submitted to the College of
Graduate Studies and Research in Partial Fulfillment
of the Requirements for the Degree of Master of Science
in the Department of Large Animal Clinical Sciences
University of Saskatchewan
Saskatoon

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ABSTRACT

Cryopreservation of germplasm is widely used in agriculture, biotechnology, conservation of threatened species and human reproductive medicine. There is a need however to improve the reproductive efficiency of breeding with cryopreserved semen, which may involve increasing the post-thaw quality of sperm through improvements in cryopreservation extenders. Extenders including egg yolk from chickens are successfully used worldwide for cryopreservation of bovine semen, whereas the protective agent in the egg yolk is believed to be the low-density lipoprotein (LDL) fraction. Egg yolks of different avian species vary in their cholesterol, phospholipid and polyunsaturated fatty acid content which have been shown to have important effects on sperm's freezing capability. The purpose of this study was to determine the cryoprotective effect of clarified egg yolk and LDLs extracted from different egg yolk sources (chicken, chicken omega-3, pigeon, quail and turkey) on bovine sperm. Semen from six bulls was collected four times each by electroejaculation, split and diluted with the 10 following extenders: chicken clarified (Ccl), chicken omega-3 clarified (O3cl), pigeon clarified (Pcl), quail clarified (Qcl), turkey clarified (Tcl), chicken LDL (CLDL), chicken omega-3 LDL (O3LDL), pigeon LDL (PLDL), quail LDL (QLDL) and turkey LDL (TLDL). The extended semen was evaluated, cryopreserved and examined directly after thawing (0h) and after two hours at 37 ˚C (2h). Computer assisted sperm analysis (CASA) was used to determine total sperm motility (TM), progressive motility (PM), straight line velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP). Intact plasma membrane (IPM) and intact acrosomes (IA) were measured by flow cytometry. The percentage change (loss; Δ%) of each sperm characteristic was calculated and used to compare the effect of the extenders. From extending to 0h post-thaw, the pigeon LDL extender lead to greater losses in sperm total and progressive motility, as well as of intact acrosomes, than the other nine extenders tested ($P < 0.05$). During 0h to 2h post-thaw, the sperm in PLDL extender experienced greater losses in total and progressive motility ($P < 0.0001$), as well as in curvilinear velocity ($P < 0.05$), than in all the other nine extenders. Sperm in turkey clarified extender had a greater loss in the velocity parameters (VSL, VAP, VCL) than sperm in several of the other extenders such as O3cl, CLDL, O3LDL, QLDL and

TLDL from 0h to 2h ($P < 0.05$). Concomitantly, sperm in the Tcl extender had a greater loss in the velocity parameters and of intact acrosomes compared to sperm in its counterpart, the turkey LDL extender, from 0h to 2h post-thaw ($P < 0.05$).

The differences produced in post-thaw quality of cryopreserved bovine sperm in the pigeon LDL and turkey clarified extenders were attributed to methodological differences in these egg yolk preparations compared with the other eight extenders.

Importantly, the results demonstrate that with most egg yolk preparations derived from a variety of species, there are equivalent cryoprotective effects afforded by the use of omega-3 chicken, pigeon, quail, or conventional chicken egg yolk in a clarified form in freezing extenders for bovine semen. We further proved that the freezing capabilities of bovine semen extenders containing the low-density lipoprotein fraction of omega-3 chicken, quail, turkey and conventional chicken egg yolk were similar.

ACKNOWLEDGEMENTS

Firstly, I have to thank Roger, Gratton, Karl, Lucky, BACA and Shake, the bulls, for finally deciding to provide me with four satisfactory samples.

I would like to thank my supervisor Dr. Claire Card for her support, patience, ideas and for everything that I learned about equine reproduction. I am grateful to my co-supervisor Dr. Carl Lessard for his guidance, help and the stimulating discussions. I also would like to express my gratitude to my committee member Dr. Muhammad Anzar for starting me down the road of flow cytometry and the insights into cryopreservation, it was much appreciated. I would like to thank my committee member Dr. Colin Palmer for his input and for keeping the focus on clinical reproduction. I am also grateful to Dr. Joseph Stookey and Dr. Reuben Mapletoft for serving as my graduate chairs and for their encouragement.

I would like to acknowledge my funding sources Westgen and Agriculture and Agri-foods Canada. Further, I am very grateful to AESB - Spring Creek, the Goodale research farm and Drs. Alvaro Garcia Guerra and Steve Hendrick for providing the bulls.

I would like to thank everyone who enabled and helped me with this project: Anne-Marie Burell, Adriane Zart, Dr. Andrea Di Marzo, Laura Hargreaves, Dr. Baljit Singh, Dr. John Campbell and Dr. Tasha Epp. I would like to extend special thanks to Lyle Boswall and Dr. Kosola Rajapaksha for their great help in the lab and to Dr. Cheryl Waldner for her excellent statistical advice.

A further special thanks to Dr. Albert Barth for passing on his profound knowledge of sperm, providing me with literature, his encouragement and for just listening. Thank you Dr. Petrie, Sue Ashburner and Jimena Yapura for your advice and your encouragement in difficult situations. Thank you Paula, Orleigh, Fernanda, Manuel, Miriam, Irfan, Dinesh and Mushtaq for being around in the lab and beyond. Thank you Nora, Ela, Rochelle, Imma and Holly for sharing the same destiny – That meant a lot to me.

Martin, Peter and Lennon - Thank you for being my family here in Saskatoon. I will never forget you!

And last but not least, a special thanks to my parents for not giving up on me after so many years of education and not stopping to cheer me up.

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ATP	Adenosine-5´-triphosphate
AI	Artificial Insemination
AV	Artificial Vagina
BSP	Binder of sperm proteins
C	Chicken
˚C	Degree Celsius
cAMP	3´-5´-cyclic adenosine monophosphate
Ccl	Chicken clarified
CFDA	Carboxyfluorescein diacetate
CLDL	Chicken low-density lipoprotein
CASA	Computer assisted sperm analysis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
E	Extended
FACS	Fluorescence activated cell sorting
FITC-PNA	Fluorescence isothiocyanate peanut agglutinin
FSC	Forward scatter
h	Hour
HOST	Hypo-osmotic swelling test
IA	Intact acrosome
IgY	Immunoglobulin Y

IPM	Intact plasma membrane
LDL(s)	Low-density lipoprotein(s)
μm	Micrometer
μl	Microliter
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
n	Number
nm	Nanometre
O3	Omega-3
O3cl	Omega-3 clarified
O3LDL	Omega-3 low-density lipoprotein
PBS	Phosphate buffered saline
P	Pigeon
P	Probability
PC	Phosphatidylcholine
Pcl	Pigeon clarified
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PI	Propidium iodide
PLDL	Pigeon low-density lipoprotein
PM	Progressive motility
PNA	Peanut agglutinin

PS	Phosphatidylserine
PSA	*Pisum sativum* agglutinin
PUFA	Polyunsaturated fatty acids
Q	Quail
Qcl	Quail clarified
QLDL	Quail low-density lipoprotein
ROS	Reactive oxygen species
SAS	Statistical analysis system
SCSA	Sperm chromatin stability assay
sec	Second(s)
SEM	Standard error of the mean
SPH	Sphingomyelin
SSC	Side scatter
T	Turkey
Tcl	Turkey clarified
TLDL	Turkey low-density lipoprotein
TM	Total motility
Tris	Tris(hydroxymethyl)aminomethane
TUNEL	Terminal transferase dUTP nick end labeling
v/v	Volume per volume
w/v	Weight per volume
ZP3	Zona pellucida glycoprotein 3

1 General Introduction

In dairy cattle, artificial insemination (AI) has replaced natural service by bulls in the Western World [1]. The latest assessment of artificial insemination worldwide quotes that around 252 million doses of frozen bovine semen and 11.6 million liquid doses of semen were produced in AI centers in 109 countries in 1998 [2]. Despite the intensive use of frozen semen in artificial insemination, there is still a need for improvement of the cryopreservation process, as about 40 to 50% of the viable sperm are damaged during freezing and thawing [3]. During cryopreservation the sperm are basically confronted with two major changes. Firstly, the decrease and increase of temperature that leads to cold and warming shocks. Secondly, the formation and dissolution of ice results in changes of osmolarity and damage due to ice crystals [4]. Cold shock can be reduced by a source of lipoproteins or high molecular weight material such as egg yolk, milk or plant-based lipids [5]. Egg yolk and milk extenders are successfully used worldwide for the cryopreservation of bovine semen [6]. Recent studies have examined the cryoprotective effect of egg yolk of different avian species and of egg yolk derived from hens fed with a diet enriched with omega-3 fatty acids [7-13]. The superiority of some egg yolk sources has been attributed to the variation in the content of cholesterol, phospholipids and polyunsaturated fatty acids [7, 9, 14]. Other components in the egg yolk may contribute to the performance of egg yolk extenders. For example, egg yolk contains several potent antioxidants such as vitamin E and phosvitin, which have a potential to inhibit chain reactive oxidation, lipid oxidation and peroxidation of the sperm membrane [15, 16]. Besides using the full egg yolk, only the low-density lipoprotein (LDL) fraction can be used, since the protective agent in the egg yolk is a phospholipid moiety of the low-density lipoprotein fraction [17-20]. A number of authors reported a positive benefit on post-thaw sperm quality when replacing the whole egg yolk by the LDL fraction [21-26]. Further, it was concluded that phosphatidylcholine, the major phospholipid in egg yolk, in the LDL is responsible for the protection of sperm during cooling and cryopreservation [27].

The egg yolks from different avian species used in the preparation of the cryopreservation extenders chosen for this study were expected to vary in their phospholipid, cholesterol and LDL composition. We compared the cryoprotective effect of clarified chicken, omega-3 chicken, pigeon, quail and turkey egg yolk in bovine semen extenders. We further

evaluated the freezing capabilities of bovine semen extenders containing the LDL fraction of omega-3 chicken, pigeon, quail, turkey and conventional chicken egg yolk.

1.1 History of artificial insemination in cattle

Research on artificial insemination (AI) dates back several centuries. Its commercial application has been around for 60 years. The first scientific step in artificial insemination (AI) was the discovery of sperm under a magnifying lens by Leeuwenhoek in the 17th century [1]. One century later, a successful artificial insemination in a dog was mentioned in a scientific publication [28]. In the 19th century, in several countries, artificial insemination was reported in the rabbit, the dog and the horse [28]. Another important step in artificial insemination was the work of the Russian physiologist Ivanov, in 1912, who not only advanced artificial insemination in the dog and the pig, but also achieved pregnancy rates in horses that were comparable to natural service [1]. Artificial vaginas (AVs) were designed for dogs, cattle, horses and sheep in Europe at the beginning of the 20th century [1]. Inspired by Ivanov, artificial insemination and related research was conducted in Japan [28]. The first book on artificial insemination was published by a British biologist in 1933 [29] and three years later, the first cooperative dairy AI organization was founded in Denmark [28]. From then on, artificial insemination in dairy cattle began to spread rapidly in the USA. The main factor for its popularity was the ability to use superior sires more efficiently. On farm safety was also an advantage, as dairy bulls were known to be dangerous and required special housing and precautions. The popularity of artificial insemination favoured the selection towards higher milk yield in dairy cattle and the national exchange of genetics. Further, artificial insemination reduced the risk of spreading venereal diseases and enabled a defined calving period [1].

Several inventions improved artificial insemination and helped with its worldwide application. This included: the development of the rectally guided transcervical method of artificial insemination in cattle by Danish veterinarians in 1937 [1] that allowed a reduction of the insemination dose; the observation that egg yolk in a buffer can preserve bovine semen for several days [30]; the discovery of glycerol which made the cryopreservation of bovine semen possible [31-33] and the addition of antibiotics to the extender to minimize bacterial contamination and limit venereal diseases [34]. On-going research lead to further improvement

and major changes in the storage of semen. The egg yolk based extender was improved, containing Tris- and citrate-buffers instead of a phosphate buffer [35]. Almquist and coworkers [36] also established an extender for bovine semen that was based on whole-milk. In 1960, liquid nitrogen replaced solid carbon dioxide dry ice allowing the long term storage of frozen semen in specialized containers [1, 28]. The use of straws instead of freezing the semen in glass ampules was an important modification to simplify the packing of cryopreserved semen [28]. On the female end, methods of detecting estrus, synchronization of estrus and timed artificial insemination made it possible to improve conception rates and therefore distribute superior genetic material worldwide [28]. Recent advances include the production of sexed semen by flow cytometry [28].

In dairy cattle, artificial insemination has replaced natural service in the Western World [1]. Due to a steady improvement in the techniques and procedures related to artificial insemination, the numbers of inseminations with one single ejaculate are amplified [1]. The latest assessment of artificial insemination worldwide quotes that around 252 million doses of frozen bovine semen and 11.6 million liquid doses were produced in AI centers in 109 countries in 1998 [2].

1.2 Sperm plasma membrane

A sperm is entirely covered by a plasma membrane, identical to somatic cells. The sperm can be divided in several membrane domains and subdomains, depending on the function [37]. The domains of the sperm head include the acrosomal and the postacrosomal region [38]. The plasma membrane of the acrosomal region can be divided into an acrosomal cap and an equatorial subdomain. The latter is separated by the posterior ring from the neck region of the midpiece [38]. Besides the specialization in function of the different domains of the plasma membrane, the lipids and proteins of the plasma membrane vary between different parts of the sperm [37].

1.2.1 Composition of sperm plasma membrane

The sperm plasma membrane consists of a phospholipid bilayer with cholesterol, complex carbohydrates and proteins, typical for plasma membranes [39]. The carbohydrate structures are bound to proteins or specific lipids on the outside of the plasma membrane (glycocalyx) [37]. The phospholipids in the sperm plasma membrane vary between mammalian species but generally include phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin, lysophosphatidylcholine and cardiolipin [40-42]. In contrast to other species, sperm from bulls have a high ratio of PC to PE [41]. The proteins constitute about 50% of the total membrane weight and can be either peripheral to or integrated in the plasma membrane [39, 43]. The cell membrane is described as a mosaic of different degrees of localized fluid areas that are also called lipid domains [44]. These domains consist of certain lipids (mainly phospholipids and sterols) with certain functions. The lipids and proteins are mobile and are able to move laterally in the plane of the membrane [45]. At room temperature, the membrane lipids are generally in a fluid (liquid crystalline) phase, but some domains contain lipids in the gel-phase [45]. In the gel-phase, the lipids are more clustered and less mobile [46]. More recent reports claimed that the proteins in the plasma membrane are predominant, cause less fluidity and therefore less lateral diffusion of the lipids [47]. Further, the degree of fluidity depends on the type and amount of the present lipids. For example, long chain polyunsaturated fatty acids in the phospholipids as well as a smaller amount of cholesterol increase the fluidity of the membrane at room temperature [48, 49].

1.2.2 Role of plasma membrane in sperm function

Concurrent with the acrosomal membrane, the sperm plasma membrane has an impact on the sperm shape and volume, motility, energy production, permeability, capacitation and acrosome reaction, and interaction with the oocyte [40]. The sperm membrane changes its lipid composition and location of the lipid domains during the physiological events before fertilization occurs [50].

1.2.2.1 Sperm Motility

Sperm motility requires adenosine triphosphate (ATP) which is produced by mitochondria (10%) and by anaerobic glycolysis in the sperm tail (90%) [51, 52]. Thus, an intact transport mechanism of monosaccharides from the extracellular environment into the sperm is essential for flagellar movement. Specific plasma membrane proteins enable the transport of glucose and fructose into the sperm [53-55]. These transporter systems require an intact plasma membrane and a specific chemical gradient of ions and other soluble components to function properly.

1.2.2.2 Changes during capacitation

The process of capacitation is still not clearly defined [39], but it is accomplished by acquisition of the ability of the sperm to fertilize an oocyte. The initial event of capacitation includes a rise in intracellular calcium, bicarbonate, and hydrogen peroxide. This triggers the production of cyclic AMP (cAMP) which in turn causes the tyrosine phosphorylation of proteins in the plasma membrane and in the cytoplasm of the sperm [39, 56]. Besides a change in conformation and dimerization of membrane proteins [37], tyrosine phosphorylation is linked to increased zona pelucida affinity [57], sperm hyperactivity [58] and the induction of the acrosome reaction [59]. Hyperactivation of the sperm occurs *in vitro* at some point during capacitation, but the processes leading to hyperactivation and acrosomal responsiveness can happen independently [60]. In addition, so-called decapacitating factors (substances coating the sperm surface) are removed from the plasma membrane during capacitation [39]. These coating factors originate from the seminal plasma and are also known as BSP (phospholipid binding proteins, bovine seminal plasma proteins or binder of sperm proteins). Their removal is essential for fertilization [27]. Another key event of capacitation is the efflux of cholesterol from the plasma membrane [37]. This leads to reorganization of lipids within the plasma membrane and in an increase in membrane fluidity [61].

1.2.2.3 Changes during acrosome reaction and fertilization

The acrosome is located underneath the sperm plasma membrane in the anterior head region and consists of a protein matrix that is enclosed by an inner and outer acrosomal membrane [39]. The conformational changes in tyrosine phosphorylated transmembrane proteins enable the binding of the sperm to the zona pelucida (ZP3 proteins) of the oocyte [37]. As a result of the phosphorylation, receptors aggregate in the sperm plasma membrane and induce a calcium ion flux into the sperm. The proteins involved form bridges between the outer acrosomal membrane and the apical sperm plasma membrane [62]. The acrosomal matrix swells and both the plasma membrane and the outer plasma membrane are dissolved by vesiculation and fusion [37]. This fusion is accomplished by the destabilization of the plasma membrane during capacitation. Subsequently, hydrolytic enzymes from the acrosomal matrix are released which dissolve the cumulus cells and the zona pelucida, and enable the penetration of the sperm into the perivitelline space of the oocyte [37].

1.3 Sperm plasma membrane changes during cryopreservation

The sperm plasma membrane is drastically altered by cryopreservation [63]. There are several mechanisms proposed how cryopreservation leads to sperm damage.

One mechanism involves lateral lipid rearrangement which destabilizes the membrane [64] (known as phase transition during cold shock). The lipid components (mainly phospholipids and sterols) of the plasma membrane undergo reorganization during the cooling process. In detail, the lipids in the bilayer start aggregating in different lipid domains which results in new associations between proteins and lipids in the membrane [64]. Therefore, the fluidity of the plasma membrane decreases during cryopreservation [50] which results in transition from liquid-crystalline to gel phase [65]. The temperature at which the phase transition occurs is specific for each particular lipid [66]. The shorter the fatty acyl chain and the more *cis*-unsaturated carbon-carbon bonds, the lower the phase transition temperature. Similarly, cholesterol decreases the phase transition temperature [66]. Freezing can further promote clustering of the lipids [64]. Rewarming of the sperm does not initially return lipids in their pre-cooling state. It was hypothesized that over time the original assembly could be achieved by lipid diffusion [64]. The

reorganization of lipids also affects the proteins, whereas integral proteins become clustered and subsequently can lose their functionality [3].

Another phenomenon related to cryopreservation is the change in membrane lipid composition [49, 63]. In boar sperm, sphingomyelin (a phospholipid) and the saturated fatty acids content of the phospholipids decreased during freezing, whereas the content of cholesterol did not change. [50]. The loss of phospholipids in sperm has also been demonstrated during rapid cooling from room temperature to 0 ˚C in several species [67], as well as during rapid freezing down to -70 ˚C [67, 68].

A third mechanism that influences the plasma membrane constitution is the peroxidation of membrane lipids as a result of formation of reactive oxygen species [63]. The high content of polyunsaturated fatty acids in the sperm membrane increases the susceptibility of sperm to peroxidation if oxygen is present [69]. The consequences of membrane peroxidation on sperm include the irreversible loss of motility, impaired metabolism, damage to the plasma membrane, leakage of intracellular enzymes, and decrease in oocyte penetration and fertilizing capacity [69].

The consequences of the changes in lipid fluidity, lipid composition and lipid damage lead to membrane destabilisation and the sperm becomes more susceptible to premature acrosome reactions [4]. Further, functional proteins like ion channel proteins are affected by the changes during cooling which is linked to a general leakiness of the sperm membrane [3]. Consequently, the decrease of sperm motility and metabolism after cooling could be attributed to the loss of cations and enzymes [61]. Since calcium channels are affected, the intracellular calcium level increases and results in a decline in sperm motility and sperm necrosis [61, 70]. The increased calcium level and the reorganization of the plasma membrane during cooling also occur during the physiologic process of capacitation [3, 61]. Further, capacitation and changes during cooling and cryopreservation of sperm have the production of reactive oxygen species and the phosphorylation of proteins in common. Due to these similarities, the damages during cryopreservation are often referred to as “capacitation-like changes” [61]. These induced alterations render the sperm to a partially capacitated state and decrease their life span.

1.4 Composition and features of extenders for bovine semen cryopreservation

During cryopreservation and thawing, the sperm are basically confronted with two major challenges. These are cold and warming shocks, and the formation and dissolution of ice which results in changes in osmolarity and damage due to ice crystals [4]. Without species specific freezing and thawing rates and diluents, the sperm would not survive the cryopreservation process. Extenders for storing cooled or cryopreserved semen have to be based on ionic or non-ionic substances that prevent changes in osmolarity and act as a buffer against changes in pH [5]. Additionally, penetrating cryoprotectants like glycerol or dimethyl sulfoxide (DMSO) and non-penetrating cryoprotectants, like glucose or fructose, reduce the intracellular ice-crystal formation. The sugars serve concurrently as an energy source. Antibiotics are generally added to minimize the growth of microorganisms originating from the seminal plasma or by contamination [5]. Furthermore, cold shock has to be antagonized and can be accomplished by a source of lipoproteins or high molecular weight material such as egg yolk, milk or plant lipids. Heated milk proved to be an appropriate diluent for cooled and frozen bovine semen [36, 71]. Freezing extenders based on milk are prepared with 10% whole milk or skim milk, 7% glycerol and antibiotics [5]. The addition of lactose to the extender enhances the cryoprotective effect of milk [72]. The casein micelles in the milk are believed to be responsible for the sperm protection during cryopreservation [27, 73]. Plant-based extenders for semen are commercially available and are based on a soybean lecithin. The protective mechanism is likely based on the binding of the lipid (soybean lecithin) to the sperm membrane [63]. Extenders that are free of components of animal origin have a lower risk of microbial contamination [74, 75]. Contaminated extenders could spread diseases or even introduce exotic diseases [76]. Further, bacterial contamination can cause production of endotoxins that are harmful to the sperm directly [77]. Compared to egg yolk extenders, the laboratory quality measures of plant-based extenders are similar [75]. However, the field fertility is lowered for soy bean extract extenders when several fertility parameters are considered in a multiphasic model [74].

1.5 Egg yolk and egg yolk extenders

The use of egg yolk in extenders dates back to 1939, when Phillips [30] discovered its protective effect on cooled bovine semen. Van Denmark et al. [78] and Foote [79] contributed to modern extender recipes by finding that 16%, 20% and 24% chicken egg yolk in the freezing extender is favourable [5, 28]. Besides using the whole egg yolk for the semen extender, a fraction with fewer particles (clarified yolk) or only the low-density lipoprotein fraction of the yolk can be used to prepare an efficient semen extender [20, 80].

1.5.1 Composition of egg yolk

Dried chicken egg yolk consists of 63% lipids and 33% proteins. The fresh egg yolk can be fractionated into 78% plasma and 22% granules. The granules contain 16% high density lipoproteins (HDL), 4% phosvitin and 2% low-density lipoproteins (LDL). In the yolk plasma, the main component is LDL (66%), followed by livetins (10%) [81]. Phosvitin is a highly phosphorylated protein with bactericidal and antioxidant properties [82]. Livetins correspond to serum proteins and are made of albumin, α-2-glycoprotein and immunoglobulin Y (IgY) [83].

Low-density lipoproteins are sphere-shaped with a liquid lipid core that is made of triglycerides and cholesterol esters. This core part is surrounded by one layer of phospholipids. Apoprotein and some cholesterols are incorporated into the phospholipid layer [84]. It is possible to fractionate low density lipoproteins into a population with a higher and a lower density (LDL_1 and LDL_2, respectively) by ultracentrifugation [85].

High-density lipoproteins were formerly known as lipovitellin and are associated with the phosvitins to form the granules. They are composed of 75 to 80% proteins and 20 to 25% lipids of which the latter contains 65% phospholipids, 30% triglycerides, and 5% cholesterol [86]. The phospholipids of the whole egg yolk are constituted of Cardiolipin, Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylserine (PS), Phosphatidylcholine (PC), and Sphingomyelin (SPH) [7].

1.5.2 Mechanism of protection by egg yolk low-density-lipoproteins

It is widely accepted that the protective agent in the egg yolk is a phospholipid moiety of the low-density lipoprotein fraction [17-20]. Thus, there are several mechanisms proposed how the LDL fraction decreases damage to the sperm during cooling and cryopreservation. One possibility is that the LDLs, particularly the phospholipids, associate with the sperm membrane and thereby provide stabilization [63, 87, 88]. Another possibility is that the phospholipids lost during sperm cryopreservation are replaced by phospholipids of the egg yolk [27, 49]. However, Quinn et al. [88] and Ricker et al. [63] did not observe that the added phospholipids were integrated into the sperm membrane. The most recent hypothesis is that the LDLs bind detrimental BSP proteins [89, 90] that are responsible for the efflux of cholesterol and phospholipids from the sperm membrane [91, 92].

Bergeron and Manjunath [27] stated that any extender containing choline phosphates is able to protect sperm during cooling and cryopreservation. Although there is evidence, that the phosphatidylcholine (component of lecithin) in the LDL fraction is the protective component, it seems that the whole lipoprotein is needed to decrease damage [5, 93]. This is supported by the fact that liposomes, artificially-prepared vesicles, made from dioleoylphosphatidylcholine, phosphatidylcholine, phosphatidylserine and combinations with cholesterol do not protect the sperm as well as the whole egg yolk [5].

1.5.3 Use of whole egg yolk, clarified egg yolk and low-density-lipoproteins extenders

An egg yolk extender provides protection for cooled and cryopreserved bovine sperm [30, 33]. It is now the most common used semen extender worldwide for bulls and contains a Tris(hydroxymethyl)aminomethane (Tris) buffer, citric acid, fructose, glycerol and 20% chicken egg yolk (v/v) [28]. This extender is also commercially available and easy to prepare.

Besides using the whole egg yolk for the semen extender, the yolk can be centrifuged and only the supernatant is mixed with the other basic components of the extender [137]; or the whole yolk can be added to the extender which is then centrifuged [80]. These clarified extenders have been successfully used for stallion and bull semen cryopreservation [80, 137]. The advantage of removing bigger particles by centrifugation is that fewer particles are interfering

with microscopic analyses and biochemical assays, whereas the sperm characteristics and the bull fertility are unchanged [80, 138].

It is believed that the agent that actually protects the sperm is the low-density lipoprotein fraction, particularly the phospholipids of the LDL [42, 139]. Several studies tested semen extenders with low-density lipoproteins and compared them to chicken egg yolk extender. In general the LDL extenders were reported to be equal or even superior to the chicken egg yolk extenders, with regard to sperm motion and structure in the bull, ram, buck, Iberian red deer, Agu pig, dog and rhesus monkey [21, 25, 26, 140-146]. In the bull, the fertilization capability of the cryopreserved sperm with LDL extender was assessed *in vitro* and *in vivo* [22, 141]. Although the cleavage rate was higher in LDL extender [22], the blastocyst formation and pregnancy rates were not different from sperm cryopreserved in chicken yolk extender.

1.5.4 Preparation of clarified and low-density-lipoprotein extenders

The preparation of clarified extenders is similar to conventional egg yolk extenders except that bigger egg yolk particles are removed. The egg yolk is either centrifuged at 600 × *g* for 10 minutes and only the supernatant is used to prepare the freezing extender [137]; or the Tris-glycerol extender containing 20% egg yolk is centrifuged at 50,000 × *g* for two hours and the supernatant is used for cryopreservation [80].

The preparation of LDL extenders involves the extraction of low-density fraction from the egg yolk. This can be achieved by several different methods that have in common the separation of the yolk components by centrifugation. The latest method was described by Moussa et al. [20] and involves basically four steps: 1) The separation of the yolk granules from the yolk plasma. The granules are soluble in sodium chloride [147] and are removed after centrifugation as the pellet [20, 93]. 2) The crucial precipitation of the livetins in the remaining egg yolk plasma, which can be achieved with ammonium sulfate. [20, 147]. The livetins are removed after centrifugation as the pellet. 3) The dialysis of the remaining supernatant (low-density lipoproteins) against distilled water to remove the ammonium sulfate and finalize the purification [20, 21]. 4) The centrifugation of the dialysate to collect the LDL fraction as the floating top layer [20]. After this stepwise extraction, the LDL extender is prepared with the

basic components of the conventional egg yolk extenders. The extender for bovine semen differs by containing 8% (w/v) LDL on dry matter basis instead of 20% (v/v) whole egg yolk [20].

1.6 Fresh and cryopreserved semen evaluation

Semen quality is one of the main factors that affect *in vivo* fertility. Laboratory sperm examination is a part of the breeding soundness evaluation, performed to select a bull for fertility and breeding [94]. The sperm analysis can be performed on fresh, cooled or frozen-thawed semen samples [95]. Several factors determine if a sperm is able to reach and successfully fertilize an oocyte, including progressive motility, active mitochondria, intact acrosome, receptors for binding to the zona pellucida and the oolemma, intact plasma membranes and a nucleus with condensed chromatin [96]. Although the routine semen analysis does not test for all these factors, new technologies allow the assessment of these parameters in a reasonable amount of time.

1.6.1 Initial semen evaluation

Immediately after the semen is collected, an initial evaluation of the sample is performed [95]. This includes the assessment of the general appearance, the volume and the concentration. The semen sample should be uniform near-white [95], but can be yellow in colour due to higher riboflavin content in some bulls [97]. The sperm concentration can be determined by the classical method using a Neubauer hemocytometer, a Makler chamber or by costlier equipment [98]. The latter includes spectrophotometric methods that rely on the absorbance or transmission of light through a solution containing sperm. Spectrophotometric methods determine the sperm concentration (photometer) or simultaneously evaluate membrane integrity (NucleoCounter). However calibration of the equipment and correction for the effect of the extender on light transmission or absorbance is essential. Further, the concentration can be assessed with a computer-assisted sperm analyzer (CASA) or fluorescence activated cell sorting (FACS) count system [98].

1.6.2 Sperm morphology

Sperm morphology is generally dependent on spermatogenesis [96] or events that occur after spermiation [99]. Poor handling techniques or problems during cooling and freezing could also damage the acrosome or cause reflection of the sperm tail [96]. Disturbances during spermatogenesis in the testis or during epididymal transit that affect sperm morphology can be classified in a variety of ways, including the spermiogram, a differential count of sperm morphology [100]. This system generates a frequency distribution of all defects. Frequent bovine defects affecting the sperm head include knobbed acrosomes, nuclear vacuoles, pyriform and detached heads. Defects that affect the midpiece such as the distal midpiece reflexes, segmental aplasia of the mitochondrial sheath (gaps), fractures, proximal droplets and teratoids are common [100]. Another system classifies sperm abnormalities based on their presumptive origin: primary defects occur during spermatogenesis and secondary defects are caused by abnormal function of the epididymis or from semen handling. A third classification system divides abnormal sperm based on their relationship to male fertility: Major sperm defects were considered to be more likely to affect male fertility and minor defects may have a minor effect on male fertility [100]. A fourth system was based on whether the defect was compensable versus uncompensable. It distinguishes between the sperm defects that can be compensated by inseminating high numbers of sperm and sperm defects that result in fertilization failure regardless of the sperm concentration inseminated [101].

Morphological evaluation can be performed visually or with computer created images. A visual subjective microscopic evaluation of sperm morphology can be conducted on unstained wet samples using differential interference phase contrast of fixed sperm or stained dried samples under oil immersion using eosin nigrosin [96]. The sperm are judged based on the percentage of normal cells and the nature of the defects as mentioned above [96]. Generally, 70% morphologically normal sperm and not more than 20% head defects are necessary to reach the highest possible fertility [102]. Computer-aided sperm head morphometric analysis uses the head length, width, and area to calculate sperm specific permutations and perimeters [96, 103]. Digital images can also be analyzed using Fourier functions which describe the shape of the sperm head [104].

1.6.3 Sperm motility and sperm motion

Sperm motility is the most commonly evaluated trait for semen quality [102]. A manual microscopic evaluation can be performed on an unstained semen sample which is evaluated for its percentage of total and/or progressively motile sperm [96]. It is an easy and fast method which does not need expensive equipment. However, the visual assessment is subjected to human bias and the repeatability within a lab and especially across labs may be of limited value [102, 105].

Alternatively to the subjective visual motility evaluation, photographic analysis or computer-assisted semen analysis (CASA) can be used [106-108]. Computer-assisted semen analysis allows the analysis of sperm concentration, sperm motility, sperm motion, and to certain extent sperm head morphology [106]. Besides the total and progressive motility, several sperm motion characteristics can be determined [109]. Some common motion characteristics include (Figure 1.1): the straight line velocity (VSL), which represents the average velocity of the sperm head measured in a straight line from the beginning to end of its track (μm/s); the average path velocity (VAP) accounts for the average point-to-point velocity of the sperm head along its average track (μm/s); and the curvilinear velocity (VCL), that defines the average point-to-point velocity of the sperm head along its actual track (μm/s) [106]. Based on these three velocity parameters, the wobble (WOB) of the sperm head, the linearity of the curvilinear track (LIN), and the straightness of the average path (STR) are calculated by the computer. The WOB (VAP/VCL) represents the degree of oscillation of the actual sperm head pathway around its average path [109]. The LIN (average of VSL/VCL in percentage) is the proximity of the sperm head's pathway to a straight line. The STR (average of VSL/VAP in percentage) estimates the proximity of the sperm head's track to a straight line with 100% being the optimal straightness. Another motion parameter is the beat cross frequency (BCF) which describes the frequency of the sperm head crossing the average path of the sperm in Hertz [106]. Further, the amplitude of the lateral head displacement (ALH, μm/s) in micrometers per second is considered an important motion parameter since it influences the outcome of *in vitro* fertilization [106, 110].

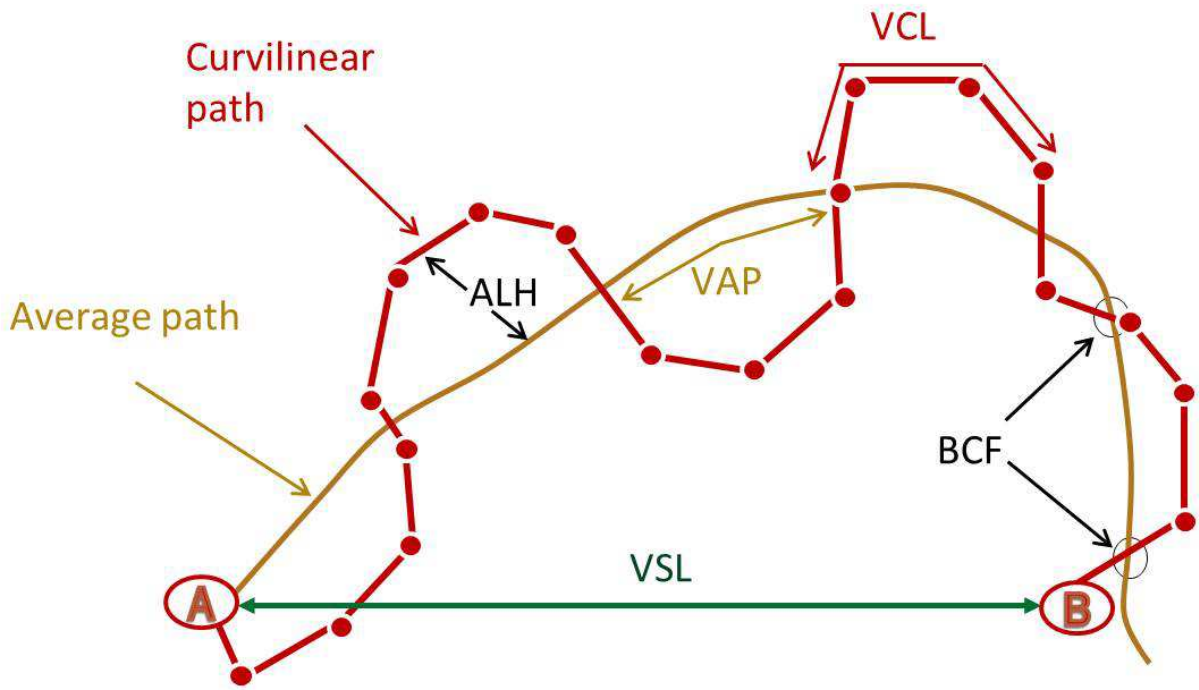

Figure 1.1 Schematic diagram of sperm kinematic measures (modified after Davis and Siemers, 1995): straigh line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), lateral head displacement (ALH) and beat cross frequency of the sperm head (BCF).

The use of CASA reduces human bias and allows for a more objective semen evaluation [106]. Similar to the manual assessment of sperm motility, there are limitations in comparing the sperm parameters across laboratories [106]. There are differences in the optics and software between machines that are on the market. The settings and parameters entered for the sperm vary along with the chamber type for the sperm and the expertise and training have an effect on the reliability, accuracy and precision of CASA. In addition, the concentration, the extender used and the temperature at which the sperm are examined has an impact on the motion parameters. Therefore, it is important to standardize and validate the operational procedures and to perform quality controls [106]. The accuracy of excluding non-sperm particles can be increased by adding fluorescent probes that label DNA [106].

1.6.4 Sperm plasma membrane permeability and integrity

Viable sperm are assessed by the percentage of sperm with an intact plasma membrane. The part of the sperm plasma membrane that covers the principal piece of a sperm can be evaluated using sperm motility or the hypo-osmotic swelling test (HOST) [95, 111, 112]. In the HOST the sperm is exposed to a hypoosmotic solution and swells due to influx of water when the cell is functionally intact, which causes a curling of the tail [112].

The plasma membrane covering the acrosome and the post-acrosomal region is assessed with a variety of stains (Trypan blue, eosin), including fluorescent dyes (PI, Hoechst 33258, CFDA, SYBR 14). These dyes either stain sperm with non-intact plasma membranes or they label sperm with intact plasma membranes. The former group of dyes includes membrane-impermeable dyes as Trypan blue and eosin, as well as fluorescence probes as propidium iodide (PI) and Hoechst 33258. The latter group contains membrane-permeable probes as carboxyfluorescein diacetate (CFDA) or SYBR 14.

The membrane-impermeable probes only diffuse through compromised plasma membranes and are known as exclusion or supravital dyes [112-114]. For example, sperm with presumably non-intact plasma membranes are stained red by the eosin of the eosin - nigrosin dye solution. Unstained cells have presumably intact plasma membranes and appear white in contrast to the dark background which is stained by the nigrosin [115]. Newer exclusion dyes are fluorescent and are excited by light at a specific wavelength to emit fluorescent light of a specific colour. The phenanthridine PI [116], the bisbenzimide Hoechst 33258 [117], YoPro-1 [118] and ethidium homodimer -1 [119] belong to this group and bind to the nucleic acids of sperm with a non-intact plasma membrane [113, 120]. Amongst these exclusion dyes, the most popular probe is PI that fluoresce red (615 to 629 nm) when excited by light of 488 nm wavelength [113]. All fluorescent dyes depend on the use of a microscope equipped with ultraviolet light, a fluorometer (automatic assessment of non-vital cells) or a flow cytometer on the basis of laser light [121].

The membrane-permeable dyes detect sperm with intact plasma membranes by diffusing into the sperm. The probes have acylated moieties which act amphipathically and the dyes are therefore able to enter the sperm [113]. Inside the sperm, the dyes are deacylated by intracellular enzymes and as a result lose their permeability [120]. The probe is thus entrapped in the living sperm, but leaks out of sperm with damaged plasma membranes. One example is CFDA, which

emits green light when trapped within an intact plasma membrane. The probe SYBR 14 binds to the DNA of the nucleus of live cells and thus clearly identifies those [120-122].

Both CFDA and SYBR 14 can be used in combination with PI of which the PI / SYBR-14 dual staining is known as the LIVE/DEAD sperm viability kit [121]: The live sperm fluoresces green due to SYBR 14 and the dead sperm emits red light due to the PI.

1.6.5 Acrosome integrity

A common method to assess the integrity of the acrosome is to use lectins that are conjugated with a fluorescence dye. In unfixed and non-permeable sperm, lectins do not have access to the acrosome; however the sperm become permeable after the acrosome reaction or after membrane disruption [113]. Consequently, the lectins gain access to the structures of the acrosome and bind to sugar moieties that are specific for the acrosome. One of the most common probes consists of *arachis hypogea* (peanut) agglutinin, a lectin from the peanut that is conjungated with fluorescein isothiocyanate (FITC). The fluorescence probe FITC emits green light at a wavelength of 515nm when excited with light of a wavelength of 488nm [113]. If the acrosome becomes disrupted or is acrosome-reacted, PNA can bind to β-galactose moieties in the outer acrosomal membrane and green fluorescence can be detected [113, 120, 121, 123]. In contrast, complete staining of the acrosome indicates an intact acrosome when the procedure is performed on fixed and permeabilized sperm [119]. When flow cytometry is used to assess the acrosome status of a sperm, PNA is preferred over others due to its selectivity for the acrosome [124]. The lectin probe *pisum sativum* agglutinin (PSA) binds to be the α-mannose and α-galactose moieties of the acrosomal matrix, but is also known for its affinity for egg yolk particles. If PSA instead of PNA is used, the sperm have to be washed to remove the egg yolk particles before using the flow cytometer [124].

A technique that also labels intact acrosomes uses Lysotracker^{TM} dyes [125]. These dyes fluoresce at pH 5.0 and accumulate in acidic organelles, such as the acrosome. Once the acrosome loses its integrity, the pH becomes neutral and the Lysotracker dye can leave the acrosome [113].

1.6.6 Mitochondrial status

It is assumed that mitochondria are the source of ATP for the sperm mid-piece and sperm head for maintaining the Na^+/K^+ gradient over the plasma membrane. This further involves the regulation of chemical and electrical gradients over the plasma membrane which sustains the plasma membrane integrity [113]. During respiration (oxidative phosphorylation), a proton gradient is built up over the inner mitochondrial membrane. The resulting potential is used by MitoTracker® probes to diffuse over the plasma membrane and the outer mitochondrial membrane and to accumulate in the inner mitochondrial membrane. Only active mitochondria will entrap the dyes. There are probes that emit light as soon as they are stimulated: MitoTracker® Deep Red 633 is excited by light with 644nm wavelength and emits red fluorescence light at 665nm wavelength [126]. Other MitoTracker® dyes like X-Rosamine do not fluorescence until they are oxidized [113]. The advantage of MitoTracker® dyes to traditional dyes like Rhodamine 123 is that they are photostable [127].

1.6.7 Other tests

There are many other tests available to assess the semen quality by laboratory means. Those include the intactness of the chromatin, changes during capacitation, apoptotic-like changes, detection of oxidative stress and lipid peroxidation [96, 120, 128].

The sperm chromatin stability assay (SCSA) is the most common assay to determine the stability of sperm chromatin [128]. For the SCSA, the sperm are treated with an acid, which may result in the exposure of single-stranded DNA, indicating instable chromatin, whereas stable DNA remains double-stranded. When the added dye acridin-orange binds to single-stranded DNA, the sperm fluoresces red (> 630 nm) when it binds to double-stranded DNA, green light (530 ± 30 nm) is emitted [128, 129]. Another DNA assay used with fluorescence or light microscopy and flow cytometry is the terminal transferase dUTP nick end labeling (TUNEL) assay that enables the detection of different degrees of DNA denaturation [120, 128].

Laboratory tests assessing the capacitation status of sperm depend on which step during capacitation is evaluated. The changes in membrane fluidity are measured with the lipid dye merocyanin 540 which is attached to a fluorescent probe like YO-PRO-1 or Hoechst 33342 [96,

120]. Hoechst 33342 binds specifically to DNA and is able to detect all sperm regardless of their membrane status. Changes in the intracellular calcium level that occur during capacitation can be assessed with chlortetracycline, Indo-1 AM or fluo-3 AM [96]. Further, changes in protein phosphorylation that are correlated with the cholesterol efflux can be measured [56]. Apoptotic-like changes that are probably caused by cryopreservation can be detected by apoptotic markers like Annexin V, YO- PRO -1 iodide and others [128]. Oxidative stress and lipid oxidation can be assessed by measuring reactive oxygen species (ROS) or superoxide anion levels by specific fluorescence dyes as 2´,7´-dichlorodihydrofluorescin or dihydroethidium, respectively. The sperm with non-intact plasma membranes are simultaneously assessed with viability dyes [120].

1.6.8 Flow cytometry

Flow cytometry can be used to analyze multiple parameters in thousands of sperm within seconds [96, 128, 130]. The sperm may be stained with one or several fluorescence dyes and used in either fresh or fixed state [121]. The sperm are then moved with additional fluid through laser beams such as argon, helium-neon or helium-cadmium in the flow cytometer. When the sperm are illuminated by a laser beam, scattered and emitted light is collected by detectors, one for the forward and one for the sideward scattered light [130, 131]. The sperm bound by the fluorochromes are excited by the specific laser beam and consequently emit fluoresce light of a certain wavelength which is detected by the specific detector [121]. Light emitted from particles other than sperm (non-sperm scatter events) are gated out and autofluorescence of the sperm is subtracted from the total fluorescence obtained [121].

The advantages of flow cytometry are that large numbers of cells may be analyzed within minutes [131], the objective assessment [132] and the possibility of using multiple probes to evaluate sperm structure and function [124, 133]. On the other hand, the equipment is expensive and requires advanced training [121].

1.6.9 Longevity

Cryopreserved semen is often evaluated after being incubated at 37 ˚C for one to four hours [102]. It is believed that the incubation imposes thermal stress to the sperm which mimics

the stress that occurs in the female tract [102]. Further, “latent” injuries from the cryopreservation process would only become apparent after the incubation time [134, 135]. It was also shown that insults originated from the sperm transition time in the epididymis did not become apparent directly after thawing, but after being incubated in the water bath [136]. However, the situation in the laboratory cannot reliably reflect the environment in the female tract and may not relate to sperm survival [135].

1.7 Relationship of laboratory assay results with sperm fertility

The goal of performing the semen assays in the laboratory is to predict the potential fertility of a given semen sample or a given bull for artificial insemination. [148].

The fertility of a sire is traditionally assessed by the non-return rates of the cows [102]. Since the non-return rate rises when the insemination dose is increased, a certain threshold of live and motile sperm is necessary to achieve maximum fertility [102, 149]. Comparisons of non-return rates with cryopreserved sperm are best determined when sperm numbers are lower, so the efficiency of fertilization at lower numbers is determined [5]. This sperm number required for maximum non-return rates is sire dependent [5]. Generally, an insemination dose should contain 8×10^6 to 10×10^6 motile sperm for most bulls or be slightly higher for bulls of below average semen quality or fertility [102].

Several studies have linked high numbers of morphologically abnormal sperm to a reduction in bull fertility [95]. Depending on the nature of the sperm defect, inseminating a high number of sperm can compensate for the morphologic abnormalities and consequently have no impact on the fertility [101]. These semen traits are described as compensable traits, whereas other sperm defects are not compensable by increasing the number [101].

Correlations between motile or progressively motile sperm and fertility of bull semen showed variations from 0.015 to 0.84 [95, 148]. Some authors reported in studies using CASA that there was little or no correlation between the non-return rates and motility [150, 151]. Other authors found that combining motility and/or several additional motion parameters resulted in high correlations [151]; especially when several fertility parameters were combined in the model [150].

The relationship between the degree of post-thaw sperm membrane damage and fertility are not clear either [148]. Januskauskas et al. [129] concluded that a single sperm viability parameter does not predict fertility (non-return rate) very reliably. Combining several independent semen traits has more power in predicting field fertility and that prediction of bull fertility is more reliable when several semen collections of one bull are assessed [129].

Regarding the acrosome, it has been shown that the ability to induce the acrosome reaction correlates highly with field fertility [152]. Performing low-dose inseminations lead to the assumption that the capability to acrosome react is an uncompensable semen trait.

However, the post-thaw semen evaluation immediately after thawing does not always correlate with bull fertility [153, 154]. The longevity test (incubation at 37˚C post-thaw) can result in better correlations of fertility with intact acrosomes [154] or semen motility [153].

In summary, the results of different studies are inconsistent regarding the correlation between laboratory test results and semen fertility. When talking about fertility, it has to be considered that the female herself and management factors (semen handling, detection of heat, insemination technique) have an enormous influence on the female fertility [95, 102]. Therefore, post-thaw semen evaluations are an analysis of the ability to cryopreserve the sperm and not about the fertility as such. However, laboratory assays are the only possibility to detect poor semen samples and exclude them from being used in artificial insemination [96].

2 Objective and Hypotheses

The objective of this study was to determine the effect of clarified egg yolk and egg yolk LDL from different sources on motility, viability and acrosome integrity of frozen-thawed bull sperm. It was hypothesized that the use of LDLs and clarified egg yolk in semen extender preserve the integrity of bovine sperm after cryopreservation. Further, the hypothesis was tested that different egg yolk sources vary in their ability to preserve the integrity of bovine sperm after cryopreservation.

3 Comparison of the cryoprotective effect of extenders based on clarified or low-density lipoprotein (LDL) preparations of egg yolk from different avian species on bovine sperm

3.1 Abstract

Cryopreservation of sperm is widely used in agriculture, biotechnology, conservation of threatened species and human reproductive medicine. The purpose of this study was to compare the cryoprotective effect of clarified and low-density lipoprotein (LDL) preparations extracted from different egg yolk sources (chicken, chicken omega-3, pigeon, quail, and turkey) on bovine sperm. Low-density lipoproteins have not been extracted from egg yolk sources other than chicken egg yolk and have not been evaluated for their cryoprotective effects. Semen was collected from six bulls four times each by electroejaculation, split and diluted in 10 different extenders. The extended semen was evaluated and cryopreserved. The sperm was examined directly after thawing (0h) and again after two hours (2h) at 37˚C. A computer assisted spermvanalysis (CASA) of total sperm motility (TM), progressive motility (PM), straight line velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP) was performed. Intact plasma membrane (IPM) and intact acrosomes (IA) were measured by flow cytometry using propidium iodide and fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA). The percentage change (loss; Δ%) of each sperm characteristic was calculated and used to compare the effect of the cryopreservation extenders. There were few differences in sperm characteristics in eight of the 10 extenders. However, pigeon LDL (PLDL) and turkey clarified (Tcl) extenders had significantly negative effects on the sperm parameters. From the addition of the extender to 0h post-thaw, PLDL extender resulted in a greater decline in the sperm characteristics TM, PM and IA than the other nine extenders ($P < 0.05$). From 0h to 2h post-thaw, the loss in sperm characteristics such as TM, PM and VCL were greater for PLDL than in the other nine extenders ($P < 0.05$). Sperm extended in Tcl had a greater decline in the following sperm characteristics: VSL, VAP, VCL and IA; compared to sperm extended in TLDL from 0h to 2h ($P < 0.05$). In summary, it was determined that cryopreservation extenders prepared with

turkey LDL, pigeon clarified, and clarified or LDL chicken, omega-3 chicken, or quail egg yolk have a similar ability to protect bovine sperm during the cryopreservation process.

3.2 Introduction

The process of sperm cryopreservation from humans, domestic and feral animals has been developed over the last 60 years [33, 155]; however there is still a need to optimize the cryopreservation process. Across all species about 40 to 50% of the viable sperm are damaged during the cryopreservation process [3]. In dairy cattle, the fertility rates with frozen semen are similar to fresh diluted semen when the amount of sperm per insemination dose is increased [5]. Increasing the proportion of viable sperm available after cryopreservation would result in a similar pregnancy rate with a reduced total sperm number per insemination dose, and would therefore increase production efficiency by producing more doses per collection [156]. The main focus of the artificial insemination industry has been on optimizing semen extenders to improve the viability of cryopreserved sperm either by replacing extender components or by supplementing existing extenders with new components [5]. Currently, one of the most common cryopreservation extenders for bovine semen contains Tris buffer, citrate, glucose and 20% chicken egg yolk (v/v) [28]. The use of egg yolk dates back to 1939, when Phillips [30] discovered its protective effect on cooled bovine semen. Van Denmark et al. [78] and Foote [79] contributed to modern extender recipes by finding that 16%, 20% and 24% egg yolk in the freezing extender was favorable [5, 28]. Further, a clarification of the egg yolk extender has been described where the egg yolk is centrifuged before or after addition to the cryopreservation extender [80, 137, 157]. The advantage of clarifying the extenders is that fewer particles interfere with microscopic analyses and biochemical assays [80, 138], whereas the sperm characteristics and the bull fertility are unchanged [138].

Conventionally, egg yolk extenders are prepared with eggs from chicken hens. However, several studies in the past decades have successfully used the egg yolk of other avian species to cryopreserve sperm from bulls, buffalos, small ruminants, boars, stallions and donkeys [7-14, 158-160].

The protective agent in the egg-yolk was found to be the lipoprotein fraction [161], particularly the LDLs [17, 18]. Since Moussa et al. [20] published a protocol which allows the

separation of egg yolk LDLs in large quantities and with 98% purity, a concentration of 8% of LDL (dry matter) in the cryopreservation extender has been found to be the optimal concentration to protect bull and buck sperm during cryopreservation [21, 22, 140]. According to Bergeron [27], phosphatidylcholine in the LDL is the essential fraction to protect the sperm during cooling and freezing.

In addition, a variety of feed supplements are used as omega-3 fatty acid sources for laying hens presumably because these additives provide additional health benefits for people [162, 163]. Chicken hens fed with these supplements produce egg yolks with measureable quantities of these additives [164, 165]. Some of these feed additives may have additional beneficial effects on sperm viability during cryopreservation [166-168].

It was hypothesized that the use of LDLs and clarified egg yolk in semen cryopreservation extenders preserves the integrity of bovine sperm after cryopreservation, and that different egg yolk sources (chicken, chicken omega-3, pigeon, quail and turkey) vary in their ability to preserve the integrity of bovine sperm after cryopreservation.

3.3 Materials and Methods

3.3.1 Extender preparation

Extenders were prepared using Triladyl® (Minitube, Ingersoll, ON, Canada) and five egg yolk sources, originated in Western Canada (Appendix A). The eggs were obtained within one week of laying and processed within one day of receiving. Each egg yolk source was used to prepare a clarified extender (cl) and a low-density lipoprotein extender (LDL). The egg yolk sources were commercially produced chicken eggs (C), commercially produced omega-3 fatty acid chicken eggs (O3), pigeon eggs from domestic privately owned pigeons (P), quail eggs from a quail operation (Q) and turkey eggs from a hatchery (T). One to two batches of each extender were prepared and stored at -80 ˚C.

3.3.1.1 Clarified extenders

Egg yolk from five different sources (C, O3, P, Q and T) was individually centrifuged for 20 min at 10,000 × *g* at 4 °C. The supernatant of the centrifuged egg yolk was used to prepare the extender with the concentrate Triladyl® (v/v). This resulted in extenders consisting of 20% clarified egg-yolk, 20% Triladyl® and 60% ultrapure water (Barnstead Nanopure™, Dubuque, IA, USA). The final concentration of glycerol in the clarified extender was 6.9%.

3.3.1.2 Low-density lipoprotein (LDL) extenders

Low-density lipoproteins were extracted from five different sources (C, O3, P, Q, T) according the method described by Moussa et al. [20] with some modifications (Appendix A). In brief, the egg yolk was diluted 1:1 with 0.9% sodium chloride (Hospira Inc. Lake Forest, IL, U.S.A.) and centrifuged twice at 10,000 × *g* for 45 min at 10˚ C to eliminate the granules and obtain the plasma (supernatant) from the egg yolk. The egg yolk livetins in the plasma were precipitated by adding 23.3 g ammonium sulphate (Sigma-Aldrich®, St. Louis, MO, USA) per 100 ml of the chicken, omega-3, quail and turkey egg yolk plasma (equivalent to a 40% saturated solution). Forty grams of ammonium sulphate were added to 100 ml of the pigeon egg yolk plasma (equivalent to a 61% saturated solution). The pH was stabilized at 8.7 and the solution was allowed to mix for one hour before the livetins were separated by centrifugation at 10,000 × *g* for 45 min at 4 ˚C. The supernatant or top layer was dialysed (Spectra/Por®, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) against distilled water for a minimum of 21 h to eliminate the ammonium sulfate and provoke the precipitation of the LDL. After centrifugation of the dialysis product at 10,000 × *g* for 45 min at 4˚ C, the floating top layer (residue) of chicken, omega-3, quail and pigeon origin was collected. Since no separation occurred in the turkey LDL preparation, the complete fraction was recovered. The amount of dry matter (DM) in the LDL was determined for each batch by drying 1 to 1.5 g of LDL at 100˚C for 48 h and calculating the percentage loss of water. Consequently, the amount of water in the LDL residue was calculated. The final LDL extender was mixed and contained 8% LDL by DM, 20% Triladyl® and 72% water (w/v). The final concentration of glycerol in the LDL extender was 6.1%.

3.3.2 Animals and semen collection

The use of the bulls and the experimental protocol was approved by the institutional animal care committee. Six beef bulls (Charolais, n = 2; Black Angus, n = 2; Red Angus, n = 1; Hereford, n = 1) between two and four years of age and from three different herds in Saskatchewan were kept at two different facilities of the WCVM during the experiment. The bulls were housed in outdoor pens and fed free choice alfalfa and grass hay and minerals. The semen was obtained by electroejaculation (Lane Pulsator IV, Lane Manufacturing, Denver, CO, USA) in winter from four bulls, in summer from one bull and in autumn from a sixth bull. Four semen samples from each bull were included in the study. The raw semen samples possessing a minimum of 70% progressive sperm motility (visual) and at least 70% morphologically normal sperm (eosin nigrosin stain) at the initial semen evaluation were selected for further processing.

3.3.3 Semen processing

The sperm were re-evaluated for motility and concentration in the laboratory with computer assisted sperm analysis (CASA). Consequently, each semen sample was diluted with the 10 different extenders at 37 ˚C to a final concentration of 50 mill sperm/ml. Within 20 minutes of extending (E) the semen, CASA and fluorescent staining were performed. The extended semen was then cooled in 15 ml Falcon™ plastic tubes (DB, Mississauga, ON, Canada) to 4 ˚C for a minimum of 120 min in a cold room. The diluted semen was manually loaded into 0.5 ml French straws (Cassou, IVM technologies, Maple Grove, MN, USA) which were sealed ultrasonically using an Ultraseal 21 (Minitube®, Ingersoll, ON, Canada) at 4 ˚C. The cooled semen straws were then processed in a computer controlled rate freezer (Icecube 14S, Sy-Lab Geräte GmbH, Neupurkdersdorf, Austria) that decreased the temperature at a rate of -2.95 ˚C/min from 4 ˚C to -10 ˚C, followed by a rate of -40 ˚C/min to -80 ˚C. The straws were then plunged into liquid nitrogen, loaded into goblets and canes and stored at -80 ˚C until post-thaw analysis.

Three straws of the same ejaculate, freezing date and extender were thawed in a 37 ˚C water bath for 30 sec, pooled and the sperm were immediately (0h) evaluated with CASA and

stained for the flow cytometry analysis. After two hours (2h) in the 37˚C water bath, the CASA evaluation and the staining was repeated.

3.3.4 Computer assisted sperm analysis (CASA)

CASA (Sperm Vision® 3.7, Minitube of America, Inc., Verona, WI, USA) was used to asses five sperm motion characteristics in 20 µm deep, "4-chamber" slides (Leja, Nieuw-Vennep, The Netherlands). Total sperm motility (TM %), progressive sperm motility (PM %), straight line velocity (VSL, the average velocity of the sperm head along a straight line from the beginning to the end of its track in µm/s), average path velocity (VAP, the average point-to-point velocity of the sperm head along its average track in µm/s), and curvilinear velocity (VCL, the average point-to-point velocity of the sperm head along its actual track in µm/s) were assessed.

3.3.5 Flow cytometry analysis

Sperm structure characteristics were evaluated simultaneously with two fluorescence dyes with flow cytometry, as previously described by Anzar et al. [169] with modifications. Propidium iodide (PI, Invitrogen, Oregon, USA; 2.4 mM in water) with excitation/emission maxima at 535/617 nm was used to assess the integrity of the sperm plasma membrane. The integrity of the acrosome was determined by excitation (488 nm) and emission (519 nm) of fluorescein isothiocyanate-conjungated peanut agglutinin (FITC-PNA, Sigma-Aldrich, St. Louis, MO, USA; stock 1 mg/ml in PBS).

A sperm-dye solution at the concentration of 1×10^6 sperm/ml was prepared by suspending 20 µl extended sperm, 6.25 µl PI, and 1 µl FITC- PNA in 1x PBS to a total volume of 1 ml for fresh extended semen. For the analysis of frozen-thawed semen, the PBS solution contained 0.5% bovine serum albumin. The solution was incubated at 37˚C for 10 min and then fixed with 10% formalin in PBS. After all samples had been stained, simultaneous fluorescence data of the probes were retrieved on 10,000 sperm per sample at a flow rate of 1.5 µl/sec on the CyFlow® Space flow cytometer (Partec GmbH, Münster, Germany). The lasers, detectors and settings were used with modifications according to the method of Hussain et al. [170]. The data were acquired by FlowMax version 2.4 software, provided by Partec. For the analysis, the

proportion of the sperm stained with PI (equivalent to cells with compromised plasma membrane) were recorded and subtracted from the total number of sperm. The resulting number of sperm was considered having intact plasma membranes (IPM). The data for sperm with intact acrosomes (IA) included sperm with and without intact plasma membranes and were retrieved from a two-dimensional FITC-PNA/PI scatter plot.

3.3.6 Statistical analysis

Statistical analyses were made using SAS statistical software (Version 9.2; SAS Institute Inc., Cary, NC USA). Descriptive statistics were performed on the actual number (Appendix B) and the relative loss (Δ%) of a sperm characteristic (TM, PM, VSL, VAP, VCL, IPM, and IA; Appendix C). The data are presented as mean ± SEM.

Chi-square analysis was used to compare the frequency of increase and decrease in TM (%) and PM (%) after dilution of the semen between pigeon LDL extender and the sum of the other nine extenders.

For the analysis with SAS Mixed Procedure, the Δ% for a selected sperm characteristic was calculated a) for the time from extending (E) to the time directly after thawing (0h) and b) for the period from 0h to 2h post-thaw.

For example, the relative loss for TM was obtained as follows:

a) TM Δ% E-0h = (TM extended – TM at 0h) / TM extended × 100……..... (3.1)

b) TM Δ% 0h-2h = (TM at 0h – TM at 2h) / TM at 0h × 100……..(3.2)

The effects of the 10 extenders on the sperm characteristics (Δ%) were compared using SAS Mixed Procedure. For each sperm characteristic, the effect of extender, collection and the interaction between extender and collection were included as fixed effects. Bull was included in the model as a random effect and the order of sample collection was accounted for as a repeated measure within bull. The post hoc analysis of means was performed using Tukey-Kramer test. Interactions between the source of egg yolk (chicken, omega-3 chicken, pigeon, quail and turkey) and the form of egg yolk preparation (clarified and LDL) were also determined with SAS Mixed Procedure: collection, source, preparation and the interaction between source and preparation were included as fixed effects. Bull was included in the model as a random effect

and the order of sample collection was accounted for as a repeated measure within bull. Differences were considered significant when $P < 0.05$.

3.4 Results

3.4.1 Changes in sperm characteristics following dilution

The overall total and progressive motility of the fresh sperm [mean ± SEM; (range)] was [78.7 ± 1.5 %; (64.0-90.7%)] and [74.1 ± 1.8 %; (58.7-88.0%)], respectively. Following the addition of the extenders the overall total and progressive motility of the sperm [mean ± SEM; (range)] was [83.3 ± 0.6 %; (46.4-95.3%)] and [77.5 ± 0.6 %; (38.5-89.7%)], respectively. For sperm in pigeon LDL extender in particular, the total and progressive motility was [71.2 ± 2.6 %; (46.4-91.7%)] and [65.4 ± 2.8 %; (38.5-88.1%)], respectively. Thus, the total motility of sperm decreased in 70.8% of cases after dilution with the pigeon LDL extender and in 20.8% of cases after dilution with any of the other nine extenders ($P < 0.05$). A decrease in progressive motility after diluting the semen with pigeon LDL extender was observed in 66.7% of cases, compared to 30.1% of cases after dilution with any of the other nine extenders ($P < 0.05$).

3.4.2 Changes in sperm characteristics during cryopreservation (Extended versus 0h)

The overall total and progressive motility of the sperm at 0h post-thaw [mean ± SEM; (range)] was [50.2 ± 0.8 %; (3.4-74.0%)] and [42.3 ± 0.8 %; (0.8-67.0%)], respectively.

The loss (Δ%) of motion (TM, PM, VSL, VAP and VCL) and structural (IPM and IA) characteristics of bovine sperm in 10 different extenders during cryopreservation is shown in Figure 3.1 and Table 3.1. Significant differences in the loss (Δ%) of sperm characteristics were obtained between some of the extenders, whereas sperm cryopreserved in PLDL and Tcl experienced several times greater losses than sperm in other extenders between extending and 0h post-thaw: The sperm cryopreserved in PLDL had greater losses in TM, PM and IA than sperm in all other extenders from E to 0h ($P < 0.05$). Sperm in PLDL showed a greater loss of VAP when compared with O3cl ($P = 0.0055$); of VCL compared with O3cl ($P = 0.0024$) and Pcl

(*P* = 0.0159); and of IPM compared to O3cl (*P* = 0.0007), Qcl *(P* = 0.0073), or Ccl (*P* = 0.0492). Sperm diluted in Tcl experienced significantly higher losses compared to sperm in O3cl in TM (*P* = 0.0361) and PM (*P* = 0.0019); and to sperm in O3cl (*P* = 0.0058) or sperm in Qcl (*P* = 0.0438) in IPM.

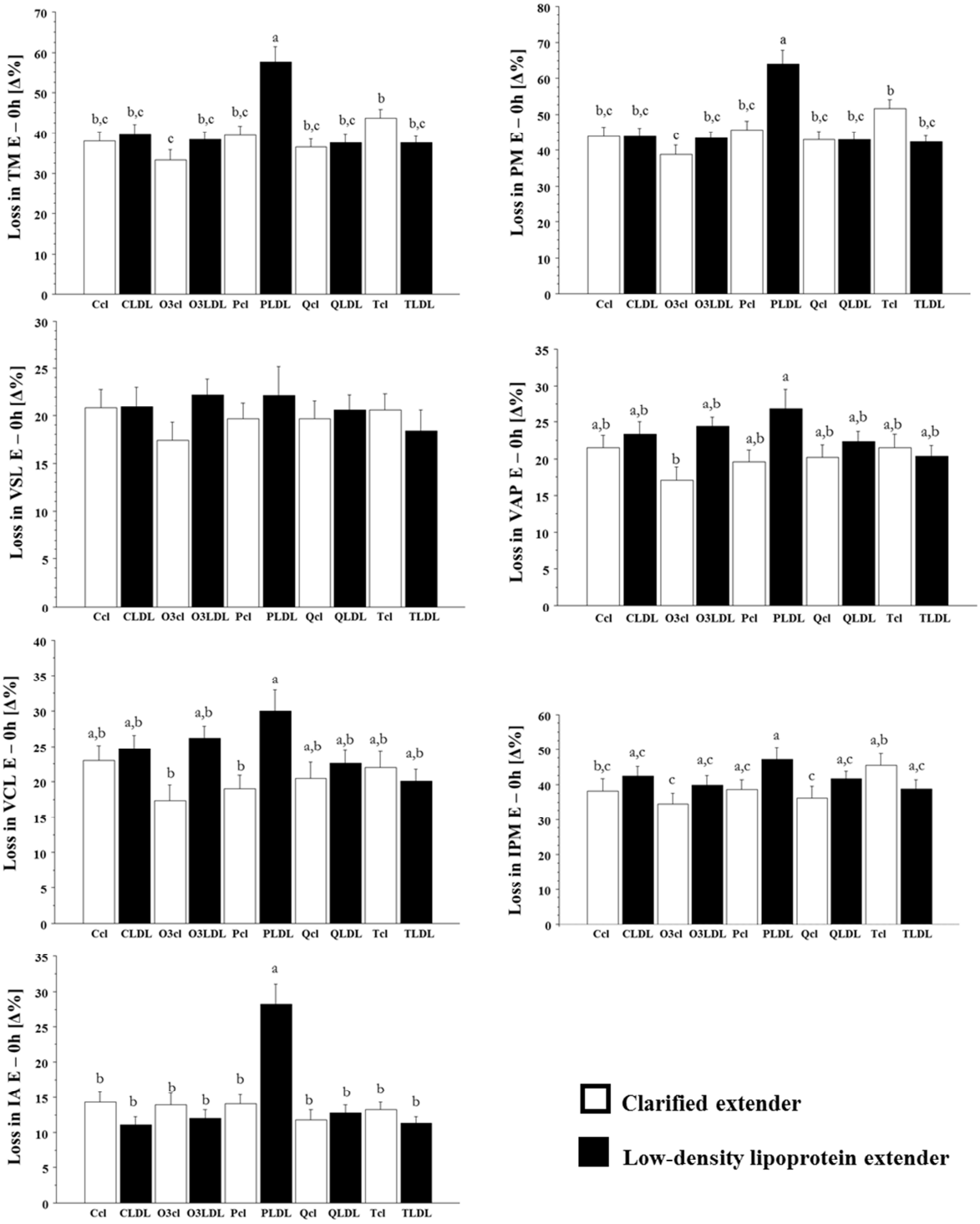

Figure 3.1 Loss (Δ%; mean ± SEM) of total motility (TM), progressive motility (PM), straight line velocity (VSL), average path velocity (VAP), curvilinear line velocity (VCL), sperm with intact plasma membrane (IPM), and sperm with intact acrosome (IA) of bovine sperm during cryopreservation (E-0h) in egg yolk extenders: chicken clarified and LDL (Ccl and CLDL), chicken omega-3 clarified and LDL

(O3cl and O3LDL), pigeon clarified and LDL (Pcl and PLDL), quail clarified and LDL (Qcl and QLDL), turkey clarified and LDL (Tcl and TLDL). Different letters indicate differences between the sperm in the extenders ($P < 0.05$); n = 24 collections.

A comparison of the losses (Δ%) between a clarified and a low-density lipoprotein extender of the same egg yolk source, revealed that sperm in the PLDL extender had greater losses in TM ($P < 0.0001$), PM ($P < 0.0001$), VCL ($P = 0.0159$) and IA ($P < 0.0001$) than sperm in the counterpart extender, Pcl, from E to 0h. There was no general comparison made between clarified against LDL extenders, since there were interactions between the egg yolk source (C, O3, P, Q, T) and the egg yolk preparation (clarified, LDL) in TM, PM, VCL, IPM and IA ($P < 0.05$).

Table 3.1 Loss (Δ%) of motion and structural characteristics of bovine sperm in 10 different cryopreservation extenders from extending to thawing (E-0h; n = 24 collections).

	Δ% E-0h						
Extender	TM	PM	VSL	VAP	VCL	IPM	IA
Ccl	37.9±2.3^{b,c}	43.9±2.6^{b,c}	20.8±1.9	21.5±1.7^{a,b}	23.0±2.1^{a,b}	38.2±3.4^{b,c}	14.3±1.5^{b}
O3cl	33.4±2.4^{c}	38.8±2.6^{c}	17.4±1.9	17.0±1.9^{b}	17.3±2.2^{b}	34.5±3.0^{c}	14.0±1.7^{b}
Pcl	39.4±2.2^{b,c}	45.7±2.3^{b,c}	19.6±1.7	19.6±1.5^{a,b}	19.0±1.9^{b}	38.6±2.9^{a,c}	14.0±1.3^{b}
Qcl	36.5±2.1^{b,c}	42.9±2.3^{b,c}	19.7±1.9	20.2±1.7^{a,b}	20.5±2.3^{a,b}	36.3±3.2^{c}	11.8±1.4^{b}
Tcl	43.8±2.1^{b}	51.6±2.4^{b}	20.5±1.8	21.5±1.8^{a,b}	22.1±2.2^{a,b}	45.6±3.2^{a,b}	13.2±1.1^{b}
CLDL	39.5±2.5^{b,c}	43.8±2.5^{b,c}	20.9±2.1	23.4±1.7^{a,b}	24.6±2.0^{a,b}	42.4±2.8^{a,c}	11.1±1.2^{b}
O3LDL	38.3±1.8^{b,c}	43.2±1.8^{b,c}	22.2±1.6	24.4±1.2^{a,b}	26.2±1.6^{a,b}	39.8±2.8^{a,c}	12.0±1.2^{b}
PLDL	57.8±3.8^{a}	64.1±3.7^{a}	22.1±3.0	26.8±2.8^{a}	30.0±3.0^{a}	47.1±3.6^{a}	28.2±2.9^{a}
QLDL	37.5±2.2^{b,c}	42.9±2.2^{b,c}	20.5±1.7	22.4±1.3^{a,b}	22.6±1.7^{a,b}	41.6±2.3^{a,c}	12.8±1.2^{b}
TLDL	37.5±1.7^{b,c}	42.4±1.6^{b,c}	18.4±2.1	20.4±1.4^{a,b}	20.1±1.7^{a,b}	38.8±2.5^{a,c}	11.3±1.0^{b}

Chicken clarified (Ccl), chicken omega-3 clarified (O3cl), pigeon clarified (Pcl), quail clarified (Qcl), turkey clarified (Tcl), chicken LDL (CLDL), chicken omega-3 LDL (O3LDL), pigeon LDL (PLDL), quail LDL (QLDL) and turkey LDL (TLDL). Total motility (TM), progressive motility (PM), straight line velocity (VSL), average path velocity (VAP), curvilinear line velocity (VCL), sperm with intact plasma membrane (IPM), and sperm with intact acrosome (IA). Data are shown as the mean ± SEM. Values with no common superscript differ within columns ($P < 0.05$).

3.4.3 Changes in sperm characteristics during incubation (0h versus 2h post-thaw)

At 2h post-thaw, the overall total and progressive motility of the sperm [mean ± SEM; (range)] was [37.8 ± 0.9 %; (1.7-68.0 %)] and [29.9 ± 0.9 %; (0.2-62.3%)], respectively.

The loss (Δ%) of motion (TM, PM, VSL, VAP and VCL) and structural (IPM and IA) characteristics of bovine sperm in 10 different cryopreservation extenders during incubation from time 0h to 2h post-thaw at 37 ˚C is shown in Figure 3.2 and Table 3.2. Sperm cryopreserved in PLDL and Tcl differed in several parameters significantly from one or more extenders from time 0h to 2h. During the two hour post-thaw incubation time, sperm diluted in PLDL had greater losses in TM, PM and VCL than sperm in the other nine extenders ($P < 0.05$). The sperm in PLDL had greater losses in VSL compared to sperm in: TLDL ($P < 0.0001$), QLDL ($P < 0.0001$), CLDL ($P = 0.0014$) or O3LDL ($P = 0.0131$); and greater losses in VAP compared to sperm in TLDL ($P < 0.0001$), QLDL ($P < 0.0001$), CLDL ($P < 0.0001$), O3LDL ($P = 0.0007$) or O3cl ($P = 0.0228$) from time 0h to 2h.

Further, sperm diluted in Tcl gained more IPM (Δ%) than sperm in eight (Ccl, O3cl, Pcl, Qcl, CLDL, O3LDL, QLDL, TLDL) of the other extenders ($P < 0.05$) during the incubation period. Sperm in Tcl experienced significantly greater losses in VSL compared to sperm in TLDL, QLDL, CLDL or O3LDL; and in VAP compared to TLDL, QLDL or CLDL from 0h to 2h post-thaw ($P < 0.05$).

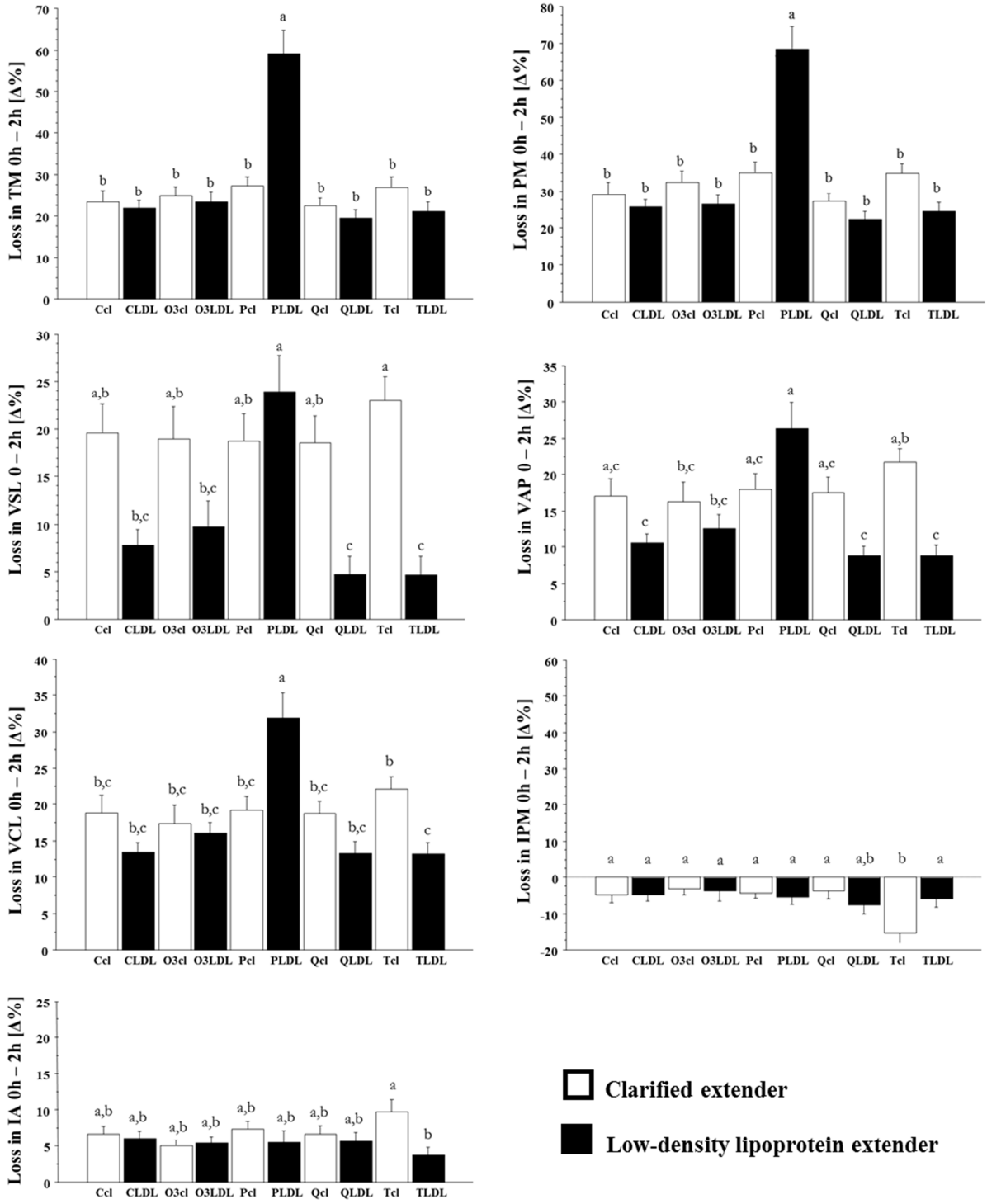

Figure 3.2 Loss (Δ%; mean ± SEM) of total motility (TM), progressive motility (PM), straight line velocity (VSL), average path velocity (VAP), curvilinear line velocity (VCL), sperm with intact plasma membrane (IPM), and sperm with intact acrosome (IA) of bovine sperm during incubation (0h-2h) in egg yolk extenders: chicken clarified and LDL (Ccl and CLDL), chicken omega-3 clarified and LDL (O3cl

and O3LDL), pigeon clarified and LDL (Pcl and PLDL), quail clarified and LDL (Qcl and QLDL), turkey clarified and LDL (Tcl and TLDL). Different letters indicate differences between the sperm in the extenders ($P < 0.05$); n = 24 collections.

A comparison of the losses (Δ%) between a clarified and a low-density lipoprotein extender of the same egg yolk source showed that sperm in the PLDL extender had greater losses in TM ($P < 0.0001$), PM ($P < 0.0001$) and VCL ($P = 0.0008$) than sperm in the counterpart extender, Pcl, from 0h to 2h. Further, a comparison of the counterpart extenders Tcl and TLDL revealed that sperm in Tcl had greater losses in VSL ($P = 0.0002$), VAP ($P = 0.0009$),VCL ($P = 0.0492$) and IA ($P = 0.0223$); but gained more IPM ($P = 0.0446$) from 0h to 2h post-thaw. There was no general comparison made between clarified against LDL extenders, since there were interactions between the egg yolk source (C, O3, P, Q, T) and the egg yolk preparation (clarified, LDL) in PM, TM, VSL, VAP, VCL and IPM ($P < 0.05$).

Table 3.2 Loss (Δ%) of motion and structural characteristics of bovine sperm in 10 different cryopreservation extenders following two hours of incubation (0h-2h) at 37˚C (n = 24 collections).

	Δ% 0h-2h						
Extender	TM	PM	VSL	VAP	VCL	IPM	IA
Ccl	23.2±2.9^{b}	29.0±3.4^{b}	19.5±3.0^{a,b}	17.0±2.4^{a,c}	18.8±2.5^{b,c}	-4.9±2.0^{a}	6.6±1.1^{a,b}
O3cl	24.8±2.3^{b}	32.3±3.1^{b}	18.9±3.4^{a,b}	16.3±2.6^{b,c}	17.3±2.5^{b,c}	-3.2±1.5^{a}	5.0±0.8^{a,b}
Pcl	27.2±2.1^{b}	34.9±2.9^{b}	18.8±2.8^{a,b}	18.0±2.1^{a,c}	19.2±2.0^{b,c}	-4.2±1.4^{a}	7.4±1.1^{a,b}
Qcl	22.3±1.9^{b}	27.1±2.2^{b}	18.6±2.8^{a,b}	17.5±2.1^{a,c}	18.6±1.8^{b,c}	-3.9±2.2^{a}	6.7±1.1^{a,b}
Tcl	26.9±2.4^{b}	34.8±2.6^{b}	23.0±2.6^{a}	21.8±1.8^{a,b}	22.1±1.7^{b}	-15.3±2.8^{b}	9.8±1.6^{a}
CLDL	21.8±1.9^{b}	25.8±1.9^{b}	7.7±1.7^{b,c}	10.6±1.2^{c}	13.5±1.2^{b,c}	-4.7±1.8^{a}	6.1±0.9^{a,b}
O3LDL	23.3±2.4^{b}	26.4±2.3^{b}	9.7±2.7^{b,c}	12.6±1.9^{b,c}	16.0±1.5^{b,c}	-3.8±2.5^{a}	5.3±1.0^{a,b}
PLDL	59.2±5.5^{a}	68.5±6.0^{a}	23.9±3.8^{a}	26.3±3.6^{a}	31.9±3.4^{a}	-5.4±2.0^{a}	5.5±1.7^{a,b}
QLDL	19.3±2.2^{b}	22.3±2.2^{b}	4.8±1.9^{c}	8.9±1.3^{c}	13.4±1.5^{b,c}	-7.5±2.4^{a,b}	5.5±1.3^{a,b}
TLDL	21.0±2.4^{b}	24.5±2.5^{b}	4.6±2.0^{c}	8.8±1.5^{c}	13.3±1.5^{c}	-6.1±2.1^{a}	3.7±1.1^{b}

Chicken clarified (Ccl), chicken omega-3 clarified (O3cl), pigeon clarified (Pcl), quail clarified (Qcl), turkey clarified (Tcl), chicken LDL (CLDL), chicken omega-3 LDL (O3LDL), pigeon LDL (PLDL), quail LDL (QLDL) and turkey LDL (TLDL). Total motility (TM), progressive motility (PM), straight line velocity (VSL), average path velocity (VAP), curvilinear line velocity (VCL), sperm with intact plasma

membrane (IPM), and sperm with intact acrosome (IA). Data are shown as the mean ± SEM. Values with no common superscript differ within columns ($P < 0.05$).

3.5 Discussion

In this study, the effects of clarified and LDL egg yolk extenders from different egg yolk sources on post-thaw sperm quality were compared. All the extenders were prepared using Triladyl® (a Tris and citric acid based glycerol extender concentrate) due to its commercial availability for veterinarians and its common use in the bovine artificial insemination industry. Our study demonstrated that LDLs extracted from chicken, omega-3 chicken, quail and turkey eggs provide equivalent protection in bovine cryopreservation semen extenders. Also, we confirmed that egg yolk from omega-3 chicken, pigeon, quail, and turkey eggs in its clarified form efficiently protects the semen during the cryopreservation procedures. This is the first study to evaluate the effects of LDLs derived from species other than chickens. In addition, this is also the first study to examine the effects of omega-3 enriched chicken egg yolk on cryopreserved bovine sperm. Under the condition of our study, it was demonstrated that the addition of pigeon LDL in a Tris-based extender could not adequately preserve the integrity of the bovine sperm after dilution and during cryopreservation.

Combining the results of the period during cryopreservation and the time after cryopreservation, differences between extenders were infrequent, except concerning the pigeon LDL and the turkey clarified extender. While there have been a number of studies comparing different egg yolk sources in semen extenders, no single egg yolk source consistently improved post-thaw semen quality [7, 9-11, 13, 14, 49, 158-160]. It has been reported that bull sperm cryopreserved in whole pigeon egg yolk yielded higher sperm motility and viability after thawing when compared to an extender prepared with chicken egg yolks [8, 12]. This is in contrast to results of Jamil-ur-Rahman et al. (2012) in which the pigeon egg yolk extender protected bull sperm equally to the chicken egg yolk extender [171]. Similarly, our study did not reveal a difference in cryoprotection between the clarified pigeon and the clarified chicken egg yolk extender.

In general, the variations observed between extenders with different egg yolk sources could be attributed to the egg yolk composition. The phospholipid, fatty acid and cholesterol content were considered to be responsible for the superiority of some egg yolk sources [7, 9, 14]. For example, better post-thaw sperm quality was attributed to an increased content of cholesterol in chicken yolk for boar semen [9] and turkey yolk for stallion semen [13]. Turkey egg yolk was reported to contain more cholesterol than chicken egg yolk [13, 172, 173], but it failed to improve the sperm quality after cryopreservation in the present study and in the Karayaka ram [10].

The addition of phosphatidylserine [66] or a higher ratio of phosphatidylcholine in the egg yolk or the LDL fraction was suggested to decrease the loss of phospholipids during the freezing and thawing process [27]. In a cryopreservation experiment on Jackass semen, whole quail egg yolk was superior to whole chicken egg yolk in protecting sperm; attributed to its higher ratio of phosphatidylcholine and polyunsaturated fatty acids [7]. However, in another study, sperm in quail whole egg yolk extender had significantly lower progressive motility and viability after cryopreservation of bull semen [12]. In the current study, both the clarified and the LDL quail extender were not different in their freezing capabilities to chicken clarified extender. Since we did not perform a biochemical analysis of the egg yolk, it was not possible to attribute differences to the cholesterol, phospholipids and polyunsaturated fatty acid (PUFA) content of the egg yolks.

Although the differences between the extenders in their cryoprotective abilities of different avian species have been linked to the variation in the lipid content in the egg yolk in several studies, it is important to consider that cholesterol, phospholipids and PUFAs are distributed all over the sperm membrane in a certain pattern [42]. The distribution pattern of the lipids and the interaction of lipids with the proteins have been suggested to be more important for the sperm function than the lipid composition itself [42]. This would explain why we and other authors observed equal cryoprotection of sperm by extenders containing different egg yolk sources.

Several studies tested chicken LDL freezing extenders that were prepared according to Moussa et al [20], using 8 % LDL (dry matter) in the extender. When compared to a chicken egg yolk extender, the chicken LDL extenders were revealed to be equal or even superior regarding sperm motion and structure in the bull, ram, buck, Iberian red deer, Agu pig, dog and rhesus

monkey [21, 25, 26, 140-146]. The current study did not reveal any differences between post-thaw qualities of sperm cryopreserved in chicken clarified or chicken LDL extender. Similarly, the clarified and the LDL forms protected bovine sperm equally in the case of extenders based on omega-3 chicken and quail eggs.

One theory about how extenders prevent cold shock and therefore damage to the sperm plasma membrane is by replacing cholesterol and phospholipids that are lost from the plasma membrane [174, 175]. These components would be reduced in the plasma membrane without the presence of an extender containing lipids or casein micelles. Bergeron et al. (2004) [89] demonstrated that there was a similar uptake of cholesterol and phosphatidylcholine into the sperm membrane over time when the semen was diluted with an extender containing the whole egg yolk or only the LDL fraction. Therefore, differences in the ability to protect sperm from damage due to cold shock between extenders containing the whole egg yolk or LDL fraction would not be expected. Our results have shown that there were no differences between the clarified extender and its counterpart LDL extender based on chicken, chicken omega-3 and quail egg yolk. This suggests that the uptake of cholesterol and phosphatidylcholine by the sperm membrane was similar during the cryopreservation process.

Another theory about how egg yolk and LDLs protect sperm during cooling and cryopreservation is based on a property of phosphatidylcholine. This phospholipid is believed to be responsible for scavenging the detrimental binder of sperm proteins (BSP proteins) from the seminal plasma [174]. The concentration of the BSP proteins and the contact time of the sperm with these proteins determine the amount of cholesterol and phospholipid efflux from the sperm membrane [91, 92]. It was concluded that if the concentration of BSP proteins was higher than the phosphatidylcholine in the extender, the sperm protection would be diminished [27]. Since the cryoprotective effect of clarified and LDL containing extenders in our study was mostly equivalent, it could be assumed that the concentration of phosphatidylcholine in both the clarified and the LDL extenders were sufficient to protect sperm.

The higher losses in sperm characteristics in the present study with pigeon LDL and turkey clarified extender are probably more related to the divergence of the preparation of these specific two extenders. The differences observed with pigeon LDL extender were more likely due to the method for the extraction of the LDL, where a higher ammonium sulfate concentration was used to precipitate the livetins. Contrary, the turkey LDL extender provided good

cryoprotection for the sperm, although the end product LDL was different in appearance than the LDL fraction of the other extenders. It could be assumed that the purity of the turkey LDL fraction was greater than in the pigeon LDL fraction. A biochemical analysis of the LDL fraction of the each egg yolk source could confirm this hypothesis. The poorer performance of the turkey clarified extender may have been caused by the larger egg yolk particles in the Tcl extender. The other four clarified and all the LDL extenders contained visually less particles. The turkey egg yolk has a thicker consistency than the egg yolk of chicken, pigeon or quail and the yolk particles were presumably not completely eliminated by the centrifugation. It is known that egg yolk particles interfere with flow cytometry assays [124] and may be interpreted as a sperm head by the CASA computer [106].

In summary, the differences between the cryoprotective effects of the extenders may not be limited to just one component like cholesterol or phoshpatidylcholine in the egg yolk, and that the variations in the method required to prepare the extender might play a greater role than a particular component or using the protective LDL fraction versus the clarified egg yolk.

In the bull, the fertilization capability of the cryopreserved sperm in LDL extender was assessed *in vitro* and *in vivo* [22, 141]. Although the embryonic cleavage rate was higher in LDL extender [22], the blastocyst formation and pregnancy rates were not different from sperm cryopreserved in clarified chicken yolk extender. The relationship between *in vitro* characteristics and *in vivo* fertility of sperm and the ability to use *in vitro* tests to predict fertility requires further investigation. Therefore, follow-up studies assessing in *in vitro* and *in vivo* fertility of the sperm in the different extenders could contribute to our understanding of fertility of the sperm in the different types of semen extenders.

In conclusion, we accepted the first part of our hypothesis that the use of LDL and clarified egg yolk in semen extenders preserves the integrity of bovine sperm after cryopreservation. However, we reject the second part that different egg yolk sources vary in their ability to preserve the integrity of bovine sperm. On the balance, the data indicate only minor differences which may have their basis on the methodology required to prepare the extenders. Based on these observations it would appear that there is currently no advantage preparing the LDL form of the egg yolk when clarified egg yolk provides a similar benefit.

4 General Discussion

Cryopreservation of sperm is of central importance to the artificial insemination industry. However, the cryopreservation of sperm is not something that nature anticipated. The cryopreservation and thawing of sperm has been shown to result in a loss of motility and membrane integrity and in a proportion of sperm, ends in cell death. The discovery of the protective effects of egg yolk and glycerol enabled sperm to survive the cryopreservation process and to preserve their fertility. These advances happened over 60 years ago and although plenty of research on cryopreservation of sperm has been performed, a considerable proportion of the sperm still does not survive or lose their fertilization ability.

In the present study, the cryoprotective capabilities of bovine semen extenders containing the clarified or low-density lipoprotein (LDL) fraction of omega-3 chicken, pigeon, quail, turkey and conventional chicken egg yolk were compared. Specifically, seven different variables (PM, TM, VSL, VAP, VCL, IPM and IA) in sperm cryopreserved in 10 different extenders (Ccl, O3cl, Pcl, Qcl, Tcl, CLDL, O3LDL, PLDL, QLDL and TLDL) after dilution, immediately after thawing and two hours after thawing were examined. Significant differences between some of the extenders were observed, whereas PLDL and Tcl had repeatedly inferior cryoprotective effects on sperm.

The components of egg yolk vary from avian species to avian species, but may also be different between breeds of one species [173, 176, 177]. Several studies have compared egg yolk from different avian species for cryoprotective properties on sperm and have observed significant differences [7-14, 158-160]. The superiority of some egg yolk sources (quail, chicken, turkey) has been attributed to the variable content of cholesterol, phospholipids and polyunsaturated fatty acids [7, 9, 14]. During cooling, cholesterol and phospholipids are removed from the sperm membrane by the binder of sperm (bovine seminal plasma) proteins [91, 92]. The high content of cholesterol in turkey egg yolk was believed to increase the progressive motility of stallion semen after freeze-thawing [14]. Loading cholesterol on cyclodextrins enabled the transfer of cholesterol into the sperm membrane and increased the post-thaw motility, viability, longevity and zona-binding capability of ram sperm [178]. Similarly, an increased cholesterol content in the sperm membrane resulted in better post-thaw bull semen quality [179, 180]. Further, it is known that species such as the rabbit or humans with a high cholesterol to phospholipid ratio in

the sperm membrane are less susceptible to cold shock than species with a low cholesterol to phospholipid ratio as the bull or the ram [178, 181]. Therefore, the reason for the success of additional cholesterol in the turkey extender could be a higher content of cholesterol in the sperm membrane that decreases the susceptibility of sperm to cold shock by lowering the phase transition temperature [65, 67]. However, the exact mechanism of sperm protection by cholesterol during cryopreservation has not yet been established [178].

Besides cholesterol, certain phospholipids have been suggested to have cryoprotective properties: Adding phosphatidylserine directly to the extender [66] or a higher ratio of phosphatidylcholine in the LDL or in the egg yolk was reported to decrease the loss of phospholipids during the freezing and thawing process [27]. Since there is an efflux of phospholipids from the sperm membrane when sperm is exposed to binder of sperm proteins, there would be two possible mechanisms how the loss of phospholipids from the sperm membrane was decreased by the addition of the extenders [42]. Firstly, the phosphatidylcholine in the lipoproteins would bind to the BSP proteins and prevent efflux of phospholipids [27, 89]. Secondly, the lost phospholipids would be replaced by phospholipids of the egg yolk or LDL extender. Indeed, Bergeron et al. [89] observed an uptake of phosphatidylcholine into the sperm membrane when sperm were exposed to egg yolk or LDL extender. In a cryopreservation experiment using donkey semen, quail egg yolk was superior to chicken egg yolk in protecting sperm, which was attributed to its higher ratio of phosphatidylcholine and polyunsaturated fatty acids (PUFAs) [7]. It was hypothesized that a high ratio of PUFAs would increase the plasma membrane integrity during cryopreservation due to an increase in membrane fluidity [49]. It has been observed that polyunsaturated fatty acids (PUFAs) are lost during cryopreservation [49] and it could be possible that they are replaced by PUFAs provided in the extender. This study could not confirm that quail or turkey clarified extender was better in its cryoprotective effects when compared to chicken egg yolk extender. Since a chemical analysis of the egg yolk was not performed, the results of this study cannot be directly attributed to the cholesterol, phospholipid and polyunsaturated fatty acid content. To make more powerful conclusions about how the extender used in this study protected the sperm, a chemical analysis of the egg yolk extenders should be performed.

Considering all parameters, it was noted that the omega-3 clarified extender yielded better results when the changes in sperm quality from dilution with the extender (E) to the

immediate post-thaw (0h) time points were compared. The omega-3 clarified treated sperm had the lowest losses by number in TM, PM, VSL, VAP, VCL and IPM from E to 0h. The differences with the sperm in the omega-3 clarified extender however were only significant when compared to sperm in extenders with the second highest and/or highest losses, and only between E to 0h. In contrast, the omega-3 LDL extender did not stand out in any of the assessed parameters, which suggests that some factor associated with the processing of the egg yolk to purify the LDL resulted in a loss of some protective functions. There is evidence that omega-3 fatty acids play a role in male reproduction [166]. The importance of omega-3 fatty acids has been demonstrated by enriching the sperm membrane with this fatty acid by changing the diet of the male animal [167, 182]. Consequently, the sperm quality improved in fresh boar semen [182] and the motion characteristics improved in frozen-thawed stallion semen [167]. Further studies with the omega-3 clarified extender should be performed to verify the positive effects. It would be also interesting to assess the cryoprotective properties of egg yolk that contains more than 75 mg omega-3 PUFA's per egg as it was used in this study.

The clarified extenders were not only investigated for their cryoprotective properties, the low-density lipoprotein fraction was also tested for similar reasons. Several studies have demonstrated that the post-thaw quality of sperm in LDL extender is equal or even superior to sperm in conventional chicken egg yolk extender [21, 25, 26, 140-146]. It was reported that the LDLs were the responsible agent in egg yolk for its cryoprotective properties [27].

Furthermore, low-density lipoproteins interfere less with laboratory assays since the larger egg yolk particles are removed during the LDL extraction process. Egg yolk particles in the extenders can interfere with laboratory assays. Using flow cytometry, egg yolk particles can have similar scatter properties as sperm and make it difficult to identify them as such [124]. When CASA is applied, the settings have to be adjusted in a way to avoid mistaking an egg yolk particle for a sperm head [106].

The results of this study confirmed that other egg yolk sources such as omega-3 chicken LDL, quail LDL and turkey LDL are similar to chicken egg yolk LDLs in their cryopreservation capabilities for bull sperm. However, differences were noted in the extraction process of the pigeon LDL, which was a suboptimal cryoprotectant. This already became obvious when the pigeon LDL extender was added to the fresh semen sample and consequently, the sperm motility parameters (TM and PM) decreased more substantially than with the other extenders. The

protocol that was used for the preparation of the LDLs, had been developed for chicken egg yolk [20]. In the present study, it was reported that the extraction protocol needed to be modified for the extraction of LDL from pigeon eggs. Further, other modifications might be needed for the extraction of the LDL fraction of turkey egg yolk, since there was no clear separation of the LDL fraction (top layer) after the last centrifugation step. It can be speculated that the reason again is that the amount of ammonium sulphate that was added needed further exploration. Therefore, another area of investigation might include the effect of different ammonium sulfate concentrations on different egg yolk sources. However, the turkey LDL extender had a similar effect on the cryopreservation of bovine semen as the chicken LDL extender. Sperm in the turkey LDL extender experienced smaller losses in VSL, VAP, VCL and IA during the two hours post-thawing than sperm in its clarified counterpart (Tcl). Turkey LDL was the only LDL extender that had fewer losses in any sperm quality parameter than the clarified extender of the same source. The sperm in the clarified extenders could not be directly compared against all sperm in the LDL extenders, since there was an interaction between the source (C, O3, P, Q and T) and the way the extender was prepared (clarified or LDL). For example, this means that the effect of a clarified extender depends on the source of the egg yolk used in the extender. Therefore it cannot be concluded that LDL extenders behave differently or similarly to clarified extenders. Considering the variation in cholesterol and other components in egg yolk between breeds of the same avian species [173], it is possible that eggs from chickens or other avian species fed different diets, or from different farms, may result in differences between the extenders.

After having discussed the reasons and variations of using egg yolks of different sources, other influences on the present results should be elucidated. It is known that egg yolk particles in the extender can interfere with flow cytometry [124] and might have affected our results. Comparing the average increase of sperm with an intact plasma membrane in all our diluents, turkey clarified extender had the maximum gain (15%) of IPMs over the two hour incubation period. The opposite would be expected, since cells lose their membrane integrity over time, which is an irreversible process, and consequently are stained by propidium iodide. This increase in IPM is biologically difficult to explain and is beyond what might be expected due to chance error associated with the use of the machine. It can be speculated that there might have been a coating effect related to the LDLs that prevented the staining of all of the IPM sperm after the

post-thaw incubation time in the water bath. This is supported by the hypothesis that the LDLs associate irreversibly with the sperm membrane [19]. The coating effect might have been enhanced during the incubation time and suppressed the contact between the PI and the sperm. Another possible explanation for the increase in IPM after thawing could be that some egg yolk particles were falsely gated as sperm. For example, the fluorescence probe PSA-FITC is known to have an affinity for egg yolk particles and to have similar scatter properties than sperm [124]. Trying to avoid this, PNA-FITC was used instead, because it binds more specifically to sperm. However, it cannot be excluded that PI was associated to egg yolk particles and those were recognized as sperm by the flow cytometer directly after thawing. During the time in the water bath, the egg yolk particles may have altered in size and surface area, so that they were not gated in the sperm region anymore. At that point, their counts would have been less than with those obtained directly after thawing. This effect may be accentuated in the turkey clarified extender since turkey egg yolk is thicker than the yolk of chicken, pigeon and quail. The turkey clarified extender in this study had visibly more egg yolk particles than the other four clarified and the LDL extenders. It is possible that not all particles were eliminated when the yolk was centrifuged for clarification. This theory implies that all LDL extenders, being clear, would have gained consistently less IPM than the clarified extenders. Since this was not the case, the gain in IPM in the other nine extenders might have been in the range of the error of the machine. The flow cytometry assay used in this study was established in our laboratory one year before the experiment began and has been applied in several studies. The validation of the assay included determining the gating region for sperm counts with Hoechst 33342. This fluorescence probe labels the DNA of cells with and without intact plasma membrane [118]. Subsequently, sperm that were considered non-viable after repeated rapid freezing in liquid nitrogen and subsequent thawing were mixed with fresh semen (presumably 75 % viability) and four ratios of life/dead sperm were prepared: 100 % alive, 50 % alive, 25 % alive and 0 % alive. These four samples were diluted with PI, FITC-PNA and MTDR and for each dye, the sperm counts (%) in the gating region were correlated with the four different proportions of the live cells. The correlation (R*) between IPM, IA and MTDR and the killed sperm was more than 0.9.

Sperm in the turkey clarified extender experienced several times more losses than sperm in some of the other extenders during cryopreservation (extended to 0h post-thaw) in motility parameters (TM, PM) and during the post-thaw period (0h to 2h) in velocity and structural

parameters (VSL, VAP, VCL, IA). It is plausible, that the amount of egg yolk particles or viscosity of the turkey clarified extender impeded the motility and velocity of the sperm and/or influenced its cryoprotective properties.

Bulls show some variability in sperm quality due to season [183]. Bulls in the experiment were collected in July, September, November and December. The losses that were observed after the time in the 37 ˚C water bath could originate from membrane damage due to the cooling, cryopreservation and thawing process [134], or they also could have originated in cell membrane damage that occurred during the sperm transition time in the epididymis [136]. These alterations in the sperm membrane do not become apparent directly after thawing, but after being incubated in the water bath [136]. Therefore some membrane damage could have occurred before the sample was ejaculated and the alterations were not detected in the fresh, the extended or directly after thawing, but only after incubation at 37 ˚C. Although the fresh semen samples had to be satisfactory to be included in the study, it is possible that the photoperiod and the cold stress caused latent sperm membrane damage. Ideally, the semen would be collected during April to July, because the semen quality is best during this time [183]. This was not feasible in this study since the animals were beef bulls used for pasture breeding during this time. The effect of season was not assessed, since the effect of sperm damage was expected to be the same for all 10 extenders within one semen sample, because one ejaculate was always split and diluted with all extenders Furthermore, the statistical model did account for the variability in season.

5 Conclusion

This study demonstrates that similar results in sperm motility and structural parameters are obtained when omega-3 chicken, pigeon, quail, or conventional chicken egg yolk in a clarified form in a cryopreservation extender for bovine semen is used. It was further shown that the cryopreservation capabilities of bovine semen extenders containing the low-density lipoprotein fraction of omega-3 chicken, quail, turkey and conventional chicken egg yolk are similar and that pigeon low-density lipoprotein extender was significantly different and resulted in suboptimal sperm motility and structural parameters.

6 Future directions

The results in this study arise from laboratory tests that might not reflect the true fertilizing ability and longevity of the sperm in the ten different extenders. Currently while there is some correlation with fertility, investigators are only able to estimate and not predict fertility with the laboratory sperm assays [148]. Further *in vitro* or *in vivo* fertilization studies are needed to confirm the positive effects of the extenders on the sperm quality. In the bull, the fertilization capability of sperm cryopreserved in chicken LDL extender was assessed *in vitro* and *in vivo* [22, 141]. Although the cleavage rate was higher in the LDL extender [22], the blastocyst and pregnancy rates were not different from sperm cryopreserved in whole chicken yolk extender. Reasons for this could be that blastocyst development depends much more on conditions prevailing during *in vitro* culture than cleaving of presumptive zygotes does [184]. Furthermore, the *in vivo* fertility is influenced by sperm dose and use of sperm of poor quality can result in normal fertility when high doses are used [185]. For further experiments, semen would be ideally tested in a breeding trial when low doses of sperm are inseminated and the fertility is measured by the first service pregnancy rate. If *in vivo* and *in vitro* fertility trials are not feasible, inducing the acrosome reaction and comparing the amount of acrosome reacted sperm between the extenders would provide additional information. It has been recently shown, that the ability to induce the acrosome reaction is a noncompensable trait that affects fertility [152].

Another option to improve egg yolk extenders other than using different sources would be to use eggs from chicken that had received a specific diet where controlled amounts of the PUFA, cholesterol, phosphatidylcholine and Vitamin E content were varied and the effect on the accumulation of those substances in the egg yolk determined. Generally, flaxseed, linseed, algae and several oils of animal or non-animal origin have been successfully used to increase the amount of polyunsaturated fatty acids (PUFAs) in egg yolk [165, 166]. Further, it is possible to enrich the egg yolk with antioxidants through a particular diet [163, 186] and specifically to counteract the propensity of low-density lipoprotein to oxidize [186]. However programmed feeding cannot increase the amount of cholesterol in the egg yolk [164, 187, 188] and is not desirable for human nutrition [173, 189, 190]. It would be interesting to test egg yolks with high cholesterol, omega-3 fatty acid and high vitamin E contents for their cryopreservation properties. However, “designing” an egg with regard to its semen freezing properties has its limitations; as

there may be health effects on the egg producing hen. Excessive flaxseed feeding to increase the PUFAs in the yolk is linked with a higher incidence of liver hemorrhage in the chickens [191].

In summary, this study did not identify an extender (clarified or LDL) that resulted in better post-thaw bovine sperm quality than the conventional clarified chicken extender. Since commercially produced chicken eggs are easily available and in most circumstances cheaper than eggs from other avian species, chicken egg yolk is still the best option. According to results of this study, a clarified omega-3 chicken extender might also be an option, provided that omega-3 chicken eggs are not significantly more expensive than chicken eggs. The disadvantage of pigeon and quail eggs is, beside their limited availability, their small size, associated with a higher effort obtaining the egg yolk. Turkey eggs are larger than chicken eggs, but in this study, the turkey clarified extender had, especially in the longevity test (2h in water bath post-thaw), higher losses in some sperm characteristics. Replacing the egg yolk by the low-density lipoprotein fraction could be an alternative if the egg yolk interferes with laboratory assays, but otherwise it is more costly and labor intensive to extract the LDLs and prepare the LDL extender.

7 References

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8 Appendix

Appendix A – Preparation of low-density-lipoprotein (LDL) extender

1. Purpose: To prepare a cryopreservation extender for bovine semen based on LDLs from egg yolk.
2. Scope: To describe a procedure for the extraction of LDL from egg yolk and for the preparation of an LDL cryopreservation extender.
3. Materials

Chicken eggs	Star Egg	Saskatoon, SK, Canada
Chicken Omega-3 chicken eggs	Star Egg	Saskatoon, SK, Canada
Pigeon eggs	Canadian Pigeon Fanciers Association; Private breeders	Saskatoon, SK, Canada
Quail eggs	Bryconn Developments Inc.	Ardrossan, AB, Canada
Turkey eggs	Charison turkey hatchery	Gunton (Winnipeg), MB, Canada
Circle filters, 150mm Ø	Whatman plc	Kent, UK
Sodium chloride (0.9%)	Hospira Inc.	Lake Forest, IL, USA
Ammonium sulphate	Sigma-Aldrich®	St. Louis, MO, USA
Dialysis Membrane, Spectra/Por®, 12-14 kD	Spectrum Laboratories, Inc.	Rancho Dominguez, CA, USA
Semen extender concentrate, Triladyl®	Minitube	Ingersoll, Ontario, Canada

4. Protocol:

1) Separation of egg yolk
    a. Clean egg shell with 70% alcohol and paper towel.

b. Break egg manually and separate egg white from egg yolk (an egg white separator can be used if needed); strain egg white into a beaker.
c. Place egg yolk on a filter (Whatman circle filters) and carefully roll the yolk on the filter to remove the remaining albumin (egg white) and the chalazas.
d. Fold a separate filter in half two times to form a pointy tip; puncture the vitelline (yolk) membrane with the filter tip; allow yolk to drain into a beaker while holding the membrane in the filter.

2) Isolation of egg yolk plasma
   a. Dilute yolk 1:1 (w/w) with isotonic saline (0.9% sodium chloride).
   b. Stir solution on magnetic stirrer for 1 h at room temperature.
   c. Centrifuge solution at 10,000 × *g* for 45 min at 10 °C.
   d. Recover supernatant and discard granules (pellet).
   e. Centrifuge supernatant at 10,000 × *g* for 45 min at 10 °C.
   f. Recover yolk plasma (supernatant) and discard granules (pellet).
3) Precipitation of livetins
   a. Work at 4 °C.
   b. Mix the yolk plasma with ammonium sulfate to a 40% saturated solution; equivalent to 23.3 g $(NH_4)2SO_4$/100 ml yolk plasma.
      MODIFICATION for PLDL: 61% ammonium sulfate solution; equivalent to 40 g $(NH_4)2SO_4$/100 ml yolk plasma.
   c. Adjust pH to 8.7 with 1M NaOH or HCl.
   d. Stir mixture on magnetic stirrer for 1 h at 4 °C.
   e. Centrifuge mixture at 10,000 × *g* for 45 min at 4 °C.
   f. Discard the livetins (pellet) and keep the supernatant.
      MODIFICATION for PLDL and TLDL: The resulting creamy (PLDL) to pasty (TLDL) top layer is used.
4) Dialysis – Elimination of ammonium sulfate
   a. Fill supernatant into a cellulose dialysis membrane (Spectra/Por 12-14 kD); close ends tightly.
   b. Dialysis in distilled water for a minimum of 21 h; change water frequently.
5) Purification of LDL

a. Centrifuge solution at 10,000 × *g* for 45 min at 4 °C.
b. Collect LDL-rich floating residue (top layer)

   MODIFICATION for PLDL: The liquid (1st batch) to creamy (2nd batch) top layer was used.

   MODIFICATION for TLDL: No separation; the entire creamy substance is used.

6) Determination of dry weight
   a. Use 1 to 1.5 g of LDL and dry it for 48 h at 100 ˚C in a drying oven.
   b. Calculate the percentage loss of water.
   c. Calculate the amount of water in the obtained LDL residue.
7) Preparation of LDL extender
   a. Calculate the weight of ingredients for the extender

      LDL: 8% (dry matter)

      Triladyl® concentrate: 20%

      Ultrapure water: 72 % ultrapure water minus % water contained in the LDL residue
   b. Mix Triladyl® and LDL on a magnetic stir at 30 °C until mixed
   c. Add water to the Triladyl®/LDL mixture and stir 30 °C until mixed

Appendix B – Sperm motion and structural characteristics (mean ± SEM), shown as actual (% and µm/s, respectively) and relative (Δ%) numbers

Legend

TM	Total motility
PM	Progressive motility
VSL	Straight line velocity
VAP	Average path velocity
VCL	Curvilinear line velocity
IPM	Intact plasma membranes
IA	Intact acrosomes
IA IPM	Intact acrosomes and intact plasma membranes
Fresh	Before extending
E	Extended (=before cryopreservation)
0h	Directly after thawing
2h	Two hours post-thaw
LDL	Low-density lipoprotein
cl	Clarified
C	Chicken
O3	Omega-3
P	Pigeon
Q	Quail
T	Turkey
Δ%	Relative change (=Loss)

TM (%)		TM (%)			TM (Δ%)	
Fresh	Extender	E	0h	2h	E-0h	0h-2h
78.7±1.5	Ccl	88.2±0.7	54.9±2.1	42.5±2.6	37.9±2.3	23.2±2.9
	O3cl	87.1±1.2	58.2±2.3	44.3±2.6	33.4±2.4	24.8±2.3
	Pcl	85.4±1.1	51.8±2.0	37.9±2.0	39.4±2.2	27.2±2.1
	Qcl	87.8±0.8	55.8±2.0	43.5±2.0	36.5±2.1	22.3±1.9
	Tcl	86.2±1.0	48.5±1.9	35.3±1.8	43.8±2.1	26.9±2.4
	CLDL	83.5±1.3	50.7±2.4	39.8±2.2	39.5±2.5	21.8±1.9
	O3LDL	80.2±2.0	49.6±2.0	38.6±2.3	38.3±1.8	23.3±2.4
	PLDL	71.2±2.6	30.3±3.1	14.6±3.6	57.8±3.8	59.2±5.5
	QLDL	81.9±1.4	51.2±2.0	41.5±2.1	37.5±2.2	19.3±2.2
	TLDL	81.3±1.6	50.9±1.6	40.4±2.0	37.5±1.7	21.0±2.4
	Total	83.3±0.6	50.2±0.8	37.8±0.9	40.2±0.8	26.9±1.1

PM (%)		PM (%)			PM (Δ%)	
Fresh	Extender	E	0h	2h	E-0h	0h-2h
74.1±1.8	Ccl	81.7±1.0	45.9±2.2	33.2±2.5	43.9±2.6	29.0±3.4
	O3cl	80.9±1.6	49.7±2.4	34.5±2.7	38.8±2.6	32.3±3.1
	Pcl	78.0±1.3	42.4±2.0	28.0±2.0	45.7±2.3	34.9±2.9
	Qcl	82.1±1.1	47.0±2.0	34.5±2.1	42.9±2.3	27.1±2.2
	Tcl	79.6±1.4	38.4±1.9	25.2±1.7	51.6±2.4	34.8±2.6
	CLDL	78.6±1.7	44.4±2.4	33.3±2.1	43.8±2.5	25.8±1.9
	O3LDL	75.4±2.2	43.1±2.0	32.3±2.2	43.2±1.8	26.4±2.3
	PLDL	65.4±2.8	24.0±3.0	10.4±3.3	64.1±3.7	68.5±6.0
	QLDL	77.0±1.6	44.0±1.9	34.6±2.0	42.9±2.2	22.3±2.2
	TLDL	76.3±1.8	44.0±1.6	33.5±1.9	42.4±1.6	24.5±2.5
	Total	77.5±0.6	42.3±0.8	29.9±0.9	45.9±0.9	32.6±1.3

VSL (μm/s)		VSL (μm/s)			VSL (Δ%)	
Fresh	Extender	E	0h	2h	E-0h	0h-2h
74.5±2.5	Ccl	48.7±1.2	38.2±0.7	30.6±1.2	20.8±1.9	19.5±3.0
	O3cl	46.5±1.4	38.0±0.8	30.6±1.3	17.4±1.9	18.9±3.4
	Pcl	47.7±1.1	38.0±0.6	30.8±1.0	19.6±1.7	18.8±2.8
	Qcl	48.4±1.2	38.5±0.6	31.2±1.0	19.7±1.9	18.6±2.8
	Tcl	49.8±1.1	39.3±0.8	30.1±1.1	20.5±1.8	23.0±2.6
	CLDL	54.3±1.3	42.5±0.9	39.1±0.8	20.9±2.1	7.7±1.7
	O3LDL	55.3±1.3	42.6±0.7	38.4±1.1	22.2±1.6	9.7±2.7
	PLDL	53.5±1.4	41.0±1.3	30.6±1.4	22.1±3.0	23.9±3.8
	QLDL	54.1±1.4	42.6±0.8	40.4±0.8	20.5±1.7	4.8±1.9
	TLDL	54.8±1.4	44.1±0.7	42.0±0.9	18.4±2.1	4.6±2.0
	Total	51.3±0.4	40.5±0.3	34.4±0.4	20.2±0.6	15.0±1.0

VAP (μm/s)		VAP (μm/s)			VAP (Δ%)	
Fresh	Extender	E	0h	2h	E-0h	0h-2h
86.9±2.2	Ccl	64.6±1.2	50.4±0.9	41.7±1.2	21.5±1.7	17.0±2.4
	O3cl	61.8±1.5	50.8±0.9	42.4±1.4	17.0±1.9	16.3±2.6
	Pcl	61.8±1.1	49.4±0.7	40.4±1.1	19.6±1.5	18.0±2.1
	Qcl	64.0±1.1	50.7±0.7	41.8±1.1	20.2±1.7	17.5±2.1
	Tcl	65.0±1.0	50.8±1.1	39.7±1.2	21.5±1.8	21.8±1.8
	CLDL	73.4±1.4	55.9±0.9	49.9±0.9	23.4±1.7	10.6±1.2
	O3LDL	74.2±1.3	55.9±0.9	48.8±1.2	24.4±1.2	12.6±1.9
	PLDL	71.8±1.5	52.0±1.7	37.6±1.7	26.8±2.8	26.3±3.6
	QLDL	72.9±1.4	56.4±1.0	51.3±0.9	22.4±1.3	8.9±1.3
	TLDL	72.2±1.3	57.2±0.7	52.1±1.0	20.4±1.4	8.8±1.5
	Total	68.2±0.5	53.0±0.4	44.6±0.5	21.7±0.6	15.8±0.8

VCL (μm/s)		VCL (μm/s)			VCL (%Δ)	
Fresh	Extender	E	0h	2h	E-0h	0h-2h
147.7±3.3	Ccl	116.7±2.9	89.1±2.2	71.9±2.3	23.0±2.1	18.8±2.5
	O3cl	110.7±3.1	90.5±2.0	74.5±2.5	17.3±2.2	17.3±2.5
	Pcl	107.7±2.6	86.4±1.7	69.6±1.8	19.0±1.9	19.2±2.0
	Qcl	113.4±2.6	89.2±1.9	72.4±1.9	20.5±2.3	18.6±1.8
	Tcl	115.0±2.6	89.2±2.6	69.0±2.0	22.1±2.2	22.1±1.7
	CLDL	138.3±3.8	103.0±2.3	88.9±1.8	24.6±2.0	13.5±1.2
	O3LDL	140.0±3.1	102.8±2.5	86.1±2.2	26.2±1.6	16.0±1.5
	PLDL	136.1±3.3	94.0±3.7	62.6±3.3	30.0±3.0	31.9±3.4
	QLDL	136.6±4.2	105.0±3.0	90.4±2.2	22.6±1.7	13.4±1.5
	TLDL	133.1±3.2	105.5±2.0	91.3±2.2	20.1±1.7	13.3±1.5
		124.8±1.3	95.5±0.9	77.7±0.9	22.6±0.7	18.4±0.7

	IPM (%)			IPM (% Δ)	
Extender	E	0h	2h	E-0h	0h-2h
Ccl	84.1±1.5	52.3±3.1	54.3±3.1	38.2±3.4	-4.9±2.0
O3cl	82.7±1.6	54.6±3.0	56.4±3.2	34.5±3.0	-3.2±1.5
Pcl	85.3±1.4	52.6±2.8	54.6±2.9	38.6±2.9	-4.2±1.4
Qcl	85.0±1.6	54.5±3.1	55.9±3.1	36.3±3.2	-3.9±2.2
Tcl	82.5±1.8	45.0±2.9	50.8±3.0	45.6±3.2	-15.3±2.8
CLDL	78.0±2.0	45.3±2.6	47.7±2.8	42.4±2.8	-4.7±1.8
O3LDL	76.4±2.5	46.0±2.6	47.6±2.8	39.8±2.8	-3.8±2.5
PLDL	63.4±4.1	33.6±3.4	35.3±3.4	47.1±3.6	-5.4±2.0
QLDL	78.1±2.0	46.2±2.6	49.6±2.7	41.6±2.3	-7.5±2.4
TLDL	78.7±2.1	48.5±2.6	50.3±2.4	38.8±2.5	-6.1±2.1
Total	79.4±0.8	47.9±1.0	50.3±1.0	40.3±1.0	-5.9±0.7

	IA (%)			IA (% Δ)	
Extender	E	0h	2h	E-0h	0h-2h
Ccl	92.4±0.6	79.4±1.6	74.3±1.9	14.3±1.5	6.6±1.07
O3cl	91.7±0.8	79.0±1.8	75.1±2.0	14.0±1.7	5.0±0.8
Pcl	93.1±0.6	80.2±1.5	74.3±1.8	14.0±1.3	7.4±1.1
Qcl	92.4±0.9	81.5±1.5	76.1±1.8	11.8±1.4	6.7±1.1
Tcl	91.8±0.9	79.8±1.5	72.0±1.9	13.2±1.1	9.8±1.6
CLDL	90.9±0.9	80.8±1.4	76.3±1.5	11.1±1.2	6.1±0.9
O3LDL	91.2±0.9	80.2±1.3	76.0±1.5	12.0±1.2	5.3±1.0
PLDL	90.5±1.2	65.2±3.0	61.6±3.0	28.2±2.9	5.5±1.7
QLDL	92.4±0.7	80.7±1.4	76.2±1.7	12.8±1.2	5.5±1.3
TLDL	93.3±0.6	82.8±1.3	79.7±1.2	11.3±1.0	3.7±1.1
Total	92.0±0.3	79.0±0.6	74.2±0.7	14.3±0.6	6.2±0.4

Appendix C – Summary of statistical analysis on Δ%

Legend

TM	Total motility
PM	Progressive motility
VSL	Straight line velocity
VAP	Average path velocity
VCL	Curvilinear line velocity
IPM	Intact plasma membranes
IA	Intact acrosomes
IA IPM	Intact acrosomes and intact plasma membranes
Fresh	Before extending
E	Extended (=before cryopreservation)
0h	Directly after thawing
2h	Two hours post-thaw
LDL	Low-density lipoprotein
cl	Clarified
C	Chicken
O3	Omega-3
P	Pigeon
Q	Quail
T	Turkey
Δ%	Relative change (= Loss)
Norm. Distr.	Normal distribution (Shapiro-Wilk)
Ext	Extender (Ccl, O3cl, Pcl, Qcl, Tcl, CLDL, O3LDL, PLDL, QLDL, TLDL)
Coll	Collection (1 to 4)
Source	Clarified or LDL
Prep	Preparation (clarified or LDL)
Diff	Difference
Evaluations	Evaluation at E, 0h or 2h
Diff evaluations	Difference between evaluations

	TM E-0h Total	TM 0h-2h Total	PM E-0h Total	PM 0h-2h Total
N	240	240	240	240
Mean [Δ%]	40.15	26.92	45.93	32.56
SD [Δ%]	13.05	17.34	13.59	19.63
SEM [Δ%]	0.84	1.12	0.88	1.27
Median [Δ%]	38.87	22.95	44.04	28.01
Range [Δ%]	80.38	92.75	83.62	101.35
Min [Δ%]	14.78	-2.90	15.26	-4.78
Max [Δ%]	95.16	89.85	98.88	96.58
Norm. Distr. [P]	<0.0001	<0.0001	<0.0001	<0.0001
Ext-Coll interaction [P]	0.9921	0.9581	0.9942	0.7503
Source-Prep interaction [P]	<0.0001	<0.0001	<0.0001	<0.0001
Influence Extender [P]	<0.0001	<0.0001	<0.0001	<0.0001
Influence Collection [P]	0.0914	0.0197	0.1071	0.0040
Diff evaluations [P]	<0.0001	0.0006	<0.0001	0.0006
Residuals homoscedasticity	Given	Given	Given	Given
Residuals Norm. Distr. [P]	0.0010	0.0005	0.0001	0.0006

	VSL E-0h Total	VSL 0h-2h Total	VAP E-0h Total	VAP 0h-2h Total
N	240	240	240	240
Mean [Δ%]	20.23	14.95	21.71	15.78
SD [Δ%]	9.82	14.96	8.83	11.71
SEM [Δ%]	0.63	0.97	0.57	0.76
Median [Δ%]	20.75	13.53	22.06	14.95
Range [Δ%]	58.69	66.44	62.21	56.85
Min [Δ%]	-2.36	-17.06	0.39	-7.39
Max [Δ%]	56.33	49.38	62.60	49.46
Norm. Distr. [P]	0.0099	0.0007	<0.0001	0.0006
Ext-Coll interaction [P]	0.4177	0.0120	0.5073	0.0021
Source-Prep interaction [P]	0.5051	0.0003	0.0842	<0.0001
Influence Extender [P]	0.7923	<0.0001	0.0104	<0.0001
Influence Collection [P]	0.0023	0.1243	<0.0001	0.0288
Diff evaluations [P]	0.0005	0.0085	<0.0001	0.0024
Residuals homoscedasticity	Given	Given	Given	Given
Residuals Norm. Distr. [P]	0.0002	0.0004	<0.0001	<0.0001

	VCL E-0h Total	VCL 0h-2h Total	IPM E-0h Total	IPM 0h-2h Total
N	240	240	239	236
Mean [Δ%]	22.56	18.43	40.29	-5.91
SD [Δ%]	10.71	11.29	14.82	10.58
SEM [Δ%]	0.69	0.73	0.96	0.69
Median [Δ%]	22.99	16.98	38.69	-5.62
Range [Δ%]	80.22	73.20	78.07	82.14
Min [Δ%]	-5.63	-8.40	2.23	-47.11
Max [Δ%]	74.59	64.80	80.29	35.03
Norm. Distr. [P]	<0.0001	<0.0001	0.0077	<0.0001
Ext-Coll interaction [P]	0.5058	0.0227	0.9782	0.1327
Source-Prep interaction [P]	0.0202	<0.0001	0.0028	0.0181
Influence Extender [P]	0.0018	<0.0001	0.0002	0.0023
Influence Collection [P]	0.0005	0.0090	0.0135	0.3101
Diff evaluations [P]	<0.0001	0.0008	0.0003	0.0302
Residuals homoscedasticity	Given	Given	Given	Given
Residuals Norm. Distr. [P]	0.0007	0.0341	0.2269	0.0010

	IA E-0h Total	IA 0h-2h Total	IA IPM E-0h Total	IA IPM 0h-2h Total
N	239	236	239	236
Mean [Δ%]	14.27	6.17	40.29	-5.83
SD [Δ%]	8.74	5.97	14.98	11.85
SEM [Δ%]	0.57	0.39	0.97	0.77
Median [Δ%]	12.73	6.01	38.70	-5.11
Range [Δ%]	56.36	35.35	83.38	132.26
Min [Δ%]	0.18	-9.61	-2.48	-93.90
Max [Δ%]	56.54	25.74	80.90	38.36
Norm. Distr. [P]	<0.0001	0.2501	0.0049	<0.0001
Ext-Coll interaction [P]	0.9565	0.9955	0.9824	0.2885
Source-Prep interaction [P]	<0.0001	0.0816	0.0021	0.0191
Influence Extender [P]	<0.0001	0.0866	<0.0001	0.0102
Influence Collection [P]	0.0002	0.0298	0.0110	0.7084
Diff evaluations [P]	0.0009	0.0009	0.0003	0.0373
Residuals homoscedasticity	Given	Given	Given	Given
Residuals Norm. Distr. [P]	<0.0001	0.2497	0.2039	<0.0001

TM E-0h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	37.91	33.39	39.42	36.46	43.75	39.55	38.35	57.77	37.46	37.48
SD [Δ%]	11.12	11.53	10.73	10.42	10.54	12.24	8.95	18.53	10.67	8.20
SEM [Δ%]	2.27	2.35	2.19	2.13	2.15	2.50	1.83	3.78	2.18	1.67
Median [Δ%]	38.24	31.71	39.60	34.70	40.92	38.12	37.64	58.68	39.02	36.87
Range [Δ%]	45.50	38.70	38.30	34.29	43.42	54.16	33.24	74.86	43.80	37.48
Min [Δ%]	18.44	16.35	23.65	21.47	28.27	21.23	24.56	20.29	14.78	17.29
Max [Δ%]	63.94	55.06	61.95	55.76	71.68	75.39	57.79	95.16	58.58	54.76
Norm. Distr. [P]	0.8226	0.0768	0.4407	0.3184	0.0074	0.1370	0.5085	0.9606	0.9959	0.8634
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

TM 0h-2h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	23.24	24.78	27.25	22.29	26.88	21.84	23.34	59.25	19.34	20.99
SD [Δ%]	14.17	11.47	10.15	9.07	11.94	9.10	11.73	26.92	10.74	11.65
SEM [Δ%]	2.89	2.34	2.07	1.85	2.44	1.86	2.39	5.49	2.19	2.38
Median [Δ%]	22.11	21.94	25.11	23.93	25.71	20.64	20.82	64.43	18.88	18.04
Range [Δ%]	60.16	36.63	35.29	34.06	45.72	42.85	53.03	85.76	48.97	47.10
Min [Δ%]	-2.90	6.47	10.31	5.56	5.37	6.40	1.48	4.09	4.37	6.89
Max [Δ%]	57.26	43.10	45.59	39.63	51.09	49.25	54.51	89.85	53.34	53.99
Norm. Distr. [P]	0.6266	0.0822	0.4367	0.7043	0.9508	0.1075	0.3688	0.0115	0.0253	0.0077
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

PM E-0h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	43.90	38.84	45.74	42.87	51.63	43.77	43.23	64.06	42.87	42.44
SD [Δ%]	12.52	12.81	11.41	11.13	11.54	12.06	8.84	18.05	10.88	7.97
SEM [Δ%]	2.56	2.61	2.33	2.27	2.36	2.46	1.80	3.69	2.22	1.63
Median [Δ%]	44.39	36.19	45.15	41.63	47.98	42.44	42.11	65.10	43.68	41.63
Range [Δ%]	58.09	45.52	43.77	36.39	47.30	57.76	34.36	72.34	41.47	36.39
Min [Δ%]	15.26	18.82	28.97	25.42	33.53	23.14	29.15	26.54	22.50	21.26
Max [Δ%]	73.34	64.34	72.74	61.81	80.83	80.90	63.51	98.88	63.96	57.65
Norm. Distr. [P]	P=0.9364	P=0.1109	P=0.3997	P=0.3212	P=0.0277	P=0.0637	P=0.6680	P=0.5516	P=0.8485	P=0.4098
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

PM 0h-2h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	28.98	32.34	34.89	27.11	34.78	25.76	26.40	68.54	22.30	24.51
SD [Δ%]	16.73	15.21	14.06	10.88	12.95	9.17	11.32	29.35	10.90	12.12
SEM [Δ%]	3.42	3.10	2.87	2.22	2.64	1.87	2.31	6.0	2.23	2.47
Median [Δ%]	25.89	29.22	35.97	28.41	36.96	26.61	23.99	76.11	21.68	20.82
Range [Δ%]	74.23	51.60	45.27	40.95	42.51	35.51	49.40	90.02	46.20	39.17
Min [Δ%]	-4.78	7.04	12.14	5.08	12.18	7.44	5.24	6.56	7.39	10.53
Max [Δ%]	69.45	58.65	57.40	46.03	54.69	42.95	54.65	96.58	53.59	49.70
Norm. Distr. [P]	0.1694	0.2920	0.2076	0.9274	0.1767	0.6362	0.6362	0.0005	0.0528	0.0157
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

VSL E-0h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	20.82	17.40	19.63	19.66	20.52	20.92	22.24	22.13	20.53	18.41
SD [Δ%]	9.33	9.36	8.20	9.34	9.01	10.27	7.96	14.87	8.37	10.46
SEM [Δ%]	1.90	1.91	1.67	1.91	1.84	2.10	1.63	3.04	1.71	2.13
Median [Δ%]	21.62	17.76	19.78	20.61	20.96	22.43	21.90	20.29	19.66	18.72
Range [Δ%]	35.97	38.87	30.64	37.08	38.21	37.25	28.97	54.78	28.19	40.99
Min [Δ%]	4.43	-2.30	7.12	5.36	3.76	2.94	7.90	1.56	7.45	-2.36
Max [Δ%]	40.40	36.56	37.76	42.43	41.98	40.19	36.87	56.33	35.63	38.63
Norm. Distr. [P]	P=0.9502	P=0.9872	P=0.5688	P=0.4589	P=0.3677	P=0.7292	P=0.6131	P=0.0107	P=0.1993	P=0.9504
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

VSL 0h-2h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	19.55	18.93	18.79	18.56	22.96	7.75	9.70	23.91	4.76	4.62
SD [Δ%]	14.82	16.77	13.69	13.53	12.57	8.42	13.28	18.70	9.16	9.75
SEM [Δ%]	3.03	3.42	2.79	2.76	2.57	1.72	2.71	3.82	1.87	1.99
Median [Δ%]	16.05	23.40	16.07	19.22	24.55	9.38	8.50	31.87	6.61	4.96
Range [Δ%]	55.36	54.90	46.18	48.03	49.24	32.48	47.86	60.19	35.22	41.04
Min [Δ%]	-6.15	-7.61	-2.42	-3.44	-4.29	-9.04	-13.40	-10.81	-15.50	-17.06
Max [Δ%]	49.21	47.29	43.75	44.59	44.94	23.44	34.46	49.38	19.72	23.98
Norm. Distr. [P]	0.8602	0.1225	0.2391	0.3469	0.8133	0.4787	0.2426	0.0210	0.3333	0.9645
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0898	0.0263	<0.0001	0.3084	0.3557

VAP E-0h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	21.53	17.02	19.60	20.19	21.55	23.35	24.40	26.75	22.35	20.39
SD [Δ%]	8.26	9.41	7.53	8.51	8.64	8.32	6.11	13.58	6.46	6.90
SEM [Δ%]	1.69	1.92	1.54	1.74	1.76	1.70	1.25	2.77	1.32	1.41
Median [Δ%]	21.63	17.60	20.56	19.97	22.05	23.31	24.35	22.81	22.22	20.84
Range [Δ%]	31.31	30.72	30.84	34.90	29.22	32.87	21.94	53.20	22.39	23.89
Min [Δ%]	3.03	0.39	2.39	6.10	6.89	7.98	13.82	9.40	10.22	8.48
Max [Δ%]	34.33	31.11	33.23	41.00	36.12	40.85	35.77	62.60	32.61	32.37
Norm. Distr. [P]	0.4367	0.2153	0.9620	0.7764	0.3898	0.9837	0.4483	0.0053	0.2302	0.3877
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

VAP 0h-2h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	17.05	16.29	18.02	17.54	21.80	10.60	12.59	26.29	8.85	8.76
SD [Δ%]	11.77	12.73	10.21	10.27	8.85	5.64	9.38	17.82	6.41	7.31
SEM [Δ%]	2.40	2.60	2.08	2.10	1.81	1.15	1.91	3.64	1.31	1.49
Median [Δ%]	16.18	18.57	16.95	19.26	23.05	9.58	12.88	32.75	9.03	9.15
Range [Δ%]	49.56	46.43	38.40	36.97	33.12	20.39	32.86	52.03	21.35	26.09
Min [Δ%]	-7.39	-6.08	0.38	-3.18	6.58	0.02	-2.97	-2.57	-0.57	-3.05
Max [Δ%]	42.17	40.35	38.78	33.79	39.70	20.41	29.89	49.46	20.78	23.04
Norm. Distr. [P]	0.9966	0.2554	0.4924	0.5497	0.6898	0.5623	0.1980	0.0093	0.3806	0.7280
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0027	0.0003	<0.0001	0.0144	0.0180

VCL E-0h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	23.01	17.33	19.04	20.52	22.06	24.63	26.24	29.99	22.64	20.13
SD [Δ%]	10.08	10.91	9.36	11.10	10.66	9.57	7.93	14.93	8.47	8.38
SEM [Δ%]	2.06	2.23	1.91	2.27	2.18	1.95	1.62	3.05	1.73	1.71
Median [Δ%]	24.06	18.22	20.90	19.59	23.24	22.26	26.08	26.49	24.74	20.55
Range [Δ%]	38.03	38.64	41.23	49.81	35.39	39.82	34.10	64.60	26.96	30.77
Min [Δ%]	1.07	-5.63	-4.78	-4.07	2.51	4.11	5.36	9.99	7.71	2.20
Max [Δ%]	39.10	33.01	36.45	45.74	37.90	43.92	39.45	74.59	34.67	32.96
Norm. Distr. [P]	0.2390	0.3822	0.5980	0.9811	0.2344	0.9195	0.6131	0.0043	0.0691	0.4974
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

VCL 0h-2h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	18.84	17.35	19.22	18.63	22.12	13.49	16.03	31.94	13.38	13.27
SD [Δ%]	12.39	12.38	9.88	8.73	8.51	5.99	7.39	16.77	7.22	7.41
SEM [Δ%]	2.53	2.53	2.02	1.78	1.74	1.22	1.51	3.42	1.5	1.5
Median [Δ%]	20.95	19.62	18.64	19.15	22.72	12.58	15.35	31.07	11.81	13.78
Range [Δ%]	53.93	47.22	39.93	33.62	31.56	26.35	24.59	63.74	26.49	31.51
Min [Δ%]	-8.40	-5.29	-0.64	-0.13	7.16	-1.28	5.87	1.06	0.32	-2.32
Max [Δ%]	45.53	41.93	39.29	33.50	38.72	25.08	30.46	64.80	26.81	29.19
Norm. Distr. [P]	0.8335	0.6809	0.9888	0.8451	0.6062	0.7391	0.1718	0.9645	0.0386	0.9988
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

IPM E-0h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	23	24	24	24	24	24	24	24	24	24
Mean [Δ%]	38.20	34.54	38.56	36.26	45.56	42.42	39.85	47.06	41.55	38.85
SD [Δ%]	16.41	14.84	14.26	15.86	15.44	13.62	13.88	17.44	11.38	12.09
SEM [Δ%]	3.42	3.03	2.91	3.24	3.15	2.78	2.83	3.56	2.32	2.47
Median [Δ%]	33.00	33.15	36.40	29.79	47.02	40.08	42.54	46.57	40.27	36.35
Range [Δ%]	62.11	49.78	55.36	45.97	62.77	53.21	65.68	66.86	42.36	46.64
Min [Δ%]	16.72	11.15	13.69	17.36	17.53	19.94	2.23	11.70	23.02	21.04
Max [Δ%]	78.83	60.93	69.05	63.32	80.29	73.15	67.90	78.55	65.38	67.68
Norm. Distr. [P]	0.0905	0.2538	0.4232	0.0082	0.7855	0.3286	0.4861	0.6257	0.2262	0.0877
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

IPM 0h-2h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	23	23	23	24	23
Mean [Δ%]	-4.87	-3.19	-4.23	-3.86	-15.25	-4.72	-3.85	-5.41	-7.54	-6.05
SD [Δ%]	9.78	7.20	6.72	10.77	13.70	8.46	11.93	9.75	11.70	10.21
SEM [Δ%]	2.00	1.47	1.37	2.20	2.80	1.76	2.49	2.03	2.39	2.13
Median [Δ%]	-4.24	-2.47	-4.10	-1.52	-14.70	-5.71	-4.64	-6.37	-6.77	-4.58
Range [Δ%]	47.55	33.73	29.96	47.75	56.06	32.49	59.25	52.83	49.38	45.69
Min [Δ%]	-37.32	-26.49	-23.67	-25.10	-47.11	-21.77	-24.22	-31.28	-30.07	-28.08
Max [Δ%]	10.23	7.24	6.30	22.65	8.95	10.72	35.03	21.55	19.31	17.61
Norm. Distr. [P]	0.0146	0.0146	0.1922	0.8402	0.6777	0.5976	0.0204	0.0383	0.5277	0.0259
Diff evaluations [P]	0.0777	0.2401	0.1246	0.1666	<0.0001	0.0848	0.1851	0.0401	0.0063	0.0323

IA E-0h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	23	24	24	24	24	24	24	24	24	24
Mean [Δ%]	14.29	13.96	14.00	11.84	13.22	11.08	12.04	28.21	12.78	11.31
SD [Δ%]	7.00	8.19	6.47	6.80	5.32	5.94	5.75	14.02	5.82	4.80
SEM [Δ%]	1.46	1.67	1.32	1.39	1.09	1.21	1.17	2.86	1.19	0.89
Median [Δ%]	13.17	13.26	41.89	11.32	13.39	11.50	12.36	28.56	12.37	11.51
Range [Δ%]	27.24	30.32	21.20	24.59	20.18	21.40	23.44	52.11	18.90	17.28
Min [Δ%]	3.40	1.70	3.19	0.18	4.73	0.91	0.79	4.43	2.96	3.70
Max [Δ%]	30.63	32.02	24.39	24.77	24.90	22.30	24.23	56.54	21.86	20.98
Norm. Distr. [P]	P=0.7010	P=0.3236	P=0.3698	P=0.2993	P=0.5459	P=0.4340	P=0.8371	P=0.6264	P=0.2351	P=0.4303
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

IA 0h-2h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	23	23	23	24	23
Mean [Δ%]	6.61	4.96	7.38	6.72	9.79	6.07	5.33	5.49	5.54	3.65
SD [Δ%]	5.25	4.05	5.32	5.54	7.94	4.50	4.86	8.16	6.42	5.18
SEM [Δ%]	1.07	0.83	1.09	1.13	1.62	0.94	1.01	1.70	1.31	1.08
Median [Δ%]	5.91	5.72	7.99	5.58	10.66	6.17	5.32	3.55	4.97	3.09
Range [Δ%]	23.22	17.82	27.58	22.14	31.20	17.90	22.99	33.79	27.99	20.54
Min [Δ%]	-5.43	-4.68	-9.37	-4.85	-9.61	-3.55	-5.46	-8.05	-6.60	-4.69
Max [Δ%]	17.79	13.14	18.21	17.29	21.59	14.35	17.53	25.74	21.40	15.84
Norm. Distr. [P]	0.8791	0.1785	0.0440	0.5426	0.2659	0.3930	0.9432	0.1997	0.8752	0.5207
Diff evaluations [P]	<0.0001	0.0009	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	0.0003	0.0002	0.0156

IA IPM E-0h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	23	24	24	24	24	24	24	24	24	24
Mean [Δ%]	37.97	34.37	38.68	36.28	45.72	42.25	39.64	47.78	41.24	38.85
SD [Δ%]	16.66	15.05	14.38	15.82	15.37	13.68	14.37	17.23	11.85	12.19
SEM [Δ%]	3.47	3.00	2.93	3.23	3.14	2.79	2.93	3.52	2.42	2.49
Median [Δ%]	34.13	33.11	36.69	29.82	46.30	40.12	42.08	47.46	39.75	36.31
Range [Δ%]	62.08	50.29	52.99	46.47	62.69	53.75	69.61	63.04	44.80	48.32
Min [Δ%]	16.95	10.73	16.77	16.76	18.21	19.67	-2.48	15.81	21.15	19.61
Max [Δ%]	79.03	61.02	69.77	63.24	80.90	73.41	67.13	78.85	65.95	67.94
Norm. Distr. [P]	0.0898	0.2437	0.2375	0.0110	0.7474	0.2586	0.2149	0.3909	0.1862	0.1230
Diff evaluations [P]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

IA IPM 0h-2h	Extender1 = Ccl	Extender2 = O3cl	Extender3 = Pcl	Extender4 = Qcl	Extender5 = Tcl	Extender6 = CLDL	Extender7 = O3LDL	Extender8 = PLDL	Extender9 = QLDL	Extender10 = TLDL
N	24	24	24	24	24	24	24	24	24	24
Mean [Δ%]	-4.10	-2.64	-3.87	-3.51	-14.13	-4.63	-3.34	-5.43	-10.65	-5.81
SD [Δ%]	9.69	7.09	7.10	10.65	13.23	8.41	12.28	9.46	20.70	10.16
SEM [Δ%]	1.98	1.45	1.45	2.17	2.70	1.75	2.56	1.97	4.22	2.12
Median [Δ%]	-2.61	-1.03	-3.49	-1.65	-13.86	-4.27	-5.36	-5.71	-5.82	-4.96
Range [Δ%]	47.54	32.61	29.28	47.87	53.66	31.06	63.69	49.11	110.62	46.91
Min [Δ%]	-36.53	-25.41	-22.49	-24.65	-43.64	-22.46	-25.33	-32.21	-93.90	-30.01
Max [Δ%]	11.01	7.19	6.79	23.23	10.02	8.60	38.36	16.90	16.72	16.90
Norm. Distr. [P]	0.0130	0.0190	0.3757	0.9102	0.8280	0.6097	0.0042	0.0787	<0.0001	0.0432
Diff evaluations [P]	0.1729	0.3735	0.1994	0.2481	<0.0001	0.1259	0.2889	0.0583	0.0004	0.0605