University of Veterinary Medicine Hannover

Effects of substituting sodium chloride and omitting antibiotics in stallion semen diluents for prolonged storage at elevated temperatures

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submitted by
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A contribution from the Virtual Center for Reproduction Medicine, Lower Saxony
Meiner Familie
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<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>AB</td>
<td>antibiotics</td>
</tr>
<tr>
<td>AI</td>
<td>artificial insemination</td>
</tr>
<tr>
<td>ALH</td>
<td>movement of lateral head displacement</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPases</td>
<td>adenosine triphosphatases</td>
</tr>
<tr>
<td>BCF</td>
<td>beat-cross frequency</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Car</td>
<td>carnitine</td>
</tr>
<tr>
<td>CASA</td>
<td>computer assisted sperm analysis</td>
</tr>
<tr>
<td>CCC</td>
<td>cation-chloride cotransporter</td>
</tr>
<tr>
<td>ChCl</td>
<td>choline chloride</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming units</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>DAS</td>
<td>Data Acquisition Software</td>
</tr>
<tr>
<td>DFI</td>
<td>DNA Fragmentation Index</td>
</tr>
<tr>
<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia</td>
</tr>
<tr>
<td>ECS</td>
<td>extracellular space</td>
</tr>
<tr>
<td>et al.</td>
<td>et alia</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acids</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH₂</td>
<td>dihydrolavlin adenine dinucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>FAO</td>
<td>fatty acid oxidation</td>
</tr>
<tr>
<td>Fig.</td>
<td>figure</td>
</tr>
<tr>
<td>FITC-PNA</td>
<td>fluorescein isothiocyanate-labeled peanut agglutinin</td>
</tr>
<tr>
<td>GAP</td>
<td>glycerol aldehyde-3-phosphate</td>
</tr>
<tr>
<td>GLUT</td>
<td>glucose transporter</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethane sulfonic acid</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est</td>
</tr>
<tr>
<td>ICS</td>
<td>intracellular space</td>
</tr>
<tr>
<td>INRA</td>
<td>Institut National de la Recherche Agronomique</td>
</tr>
<tr>
<td>JC-1</td>
<td>tetrachloro-tetraethylbenzimidzolyl carbocianyne iodine</td>
</tr>
<tr>
<td>KCC</td>
<td>$K^+$/Cl$^-$ co-transporter</td>
</tr>
<tr>
<td>LDH-C</td>
<td>lactate dehydrogenase C</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoproteins</td>
</tr>
<tr>
<td>LIN</td>
<td>linearity</td>
</tr>
<tr>
<td>LN$_2$</td>
<td>liquid nitrogen</td>
</tr>
<tr>
<td>m-eBWWMM</td>
<td>modified equine Biggers, Whitten and Whittingham medium</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MMP</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>mOsm</td>
<td>milliosmole</td>
</tr>
<tr>
<td>mW</td>
<td>megawatt</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>nicotinamide adenine dinucleotide ion</td>
</tr>
<tr>
<td>NADH</td>
<td>1,4-dihydrnicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NHE</td>
<td>Na$^+$/H$^+$ exchanger</td>
</tr>
<tr>
<td>NKCC</td>
<td>Na$^+$-K$^+$-2Cl$^-$ cotransporter</td>
</tr>
<tr>
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<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>nM</td>
<td>nanometer</td>
</tr>
<tr>
<td>NOX5</td>
<td>membrane-associated NADPH oxidase 5</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
</tr>
<tr>
<td>PG</td>
<td>see SG</td>
</tr>
<tr>
<td>pH</td>
<td>potentia hydrogenii</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PPARG</td>
<td>peroxisome proliferator-activation receptor gamma</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>r.u.</td>
<td>relative units</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SCSA</td>
<td>sperm chromatin structure assay</td>
</tr>
<tr>
<td>SG</td>
<td>sodium green</td>
</tr>
<tr>
<td>SMP</td>
<td>skim milk powder</td>
</tr>
<tr>
<td>sNHE</td>
<td>sperm-specific isoform of NHE</td>
</tr>
<tr>
<td>spp.</td>
<td>subspecies</td>
</tr>
<tr>
<td>STR</td>
<td>straightness</td>
</tr>
<tr>
<td>Tab.</td>
<td>table</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>VAP</td>
<td>velocity average path</td>
</tr>
<tr>
<td>VCL</td>
<td>curvilinear velocity</td>
</tr>
<tr>
<td>VSL</td>
<td>straight-line velocity</td>
</tr>
<tr>
<td>w/v</td>
<td>weight by volume</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
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</table>
1 INTRODUCTION: AIMS AND OUTLINE

In the horse breeding industry, most of the warmblood mares are inseminated via artificial insemination. Raw semen can be used only for a limited period of time for artificial insemination. Sperm longevity can be increased when diluting in specific ‘extenders’ and when stored at lower temperatures where the metabolism is reduced. Cooling sperm to 5°C, however, causes cold shock and related damage. Typical extenders are composed of physiological salts, nutrients, buffering compounds, protective agents and agents that counteract microbial growth (i.e. antibiotics and antimycotics).

Increased intracellular sodium concentrations result in the activation of Na\(^+\)-K\(^+\)-ATPases to maintain homeostasis. This coincides with ATP consumption. Use of a reduced sodium medium, in which NaCl is substituted by e.g. ChCl, may be beneficial. Moreover, adding (alternative) energy sources (i.e. L-carnitine, L-histidine) may also overcome depletion of ATP during storage. Recently a modified BWWM medium has been developed for equine semen, in which rosiglitazone was added (SWEGEN et al. 2016) and NaCl was replaced by L-carnitine/L-histidine (GIBB et al. 2016). The authors claim that this diluent can be used for long term storage at elevated temperatures.

In this study, the efficacy of several modified equine BWW media (m-eBWWM) was evaluated; with special emphasis on replacing NaCl by other components and stallion sperm viability and fertility at different storage temperatures. In addition, the need for adding antibiotics was investigated. The aims of the studies described in this thesis were to: (1) investigate sperm viability during storage in m-eBWWM of a
defined osmolality with varying NaCl, ChCl, L-histidine and L-carnitine concentrations, (2) determine effects of adding skim milk powder, coenzyme Q10 and polyvinyl alcohol to m-eBWWM, (3) investigate stallion sperm viability during storage for up to 7 days (d) at temperatures ranging from 5–30°C, and (4) determine if diluents without antibiotics can be used for storing stallion semen at 5 and 17°C.
2 LITERATURE STUDY

2.1 Sperm diluents: buffering compounds, nutrients and energy sources

Fresh semen can be used for artificial insemination only for a limited period of time. Semen, however, can be preserved by dilution with specific diluents which are referred to as ‘extenders’. A selection of commonly used extender is shown in table 2.1. Diluting semen with an extender prolongs sperm viability, and insemination doses can be stored for up to 3 d if stored at 5°C. To facilitate this, semen extenders include different compounds. Typical extenders are composed of physiological salts, nutrients, buffering compounds, protective agents, and agents that counteract microbial growth (i.e. antibiotics and antimycotics). In general, they have a pH ranging from 6.7–7.2 and osmolality of 300–360 mOsm kg⁻¹. Sugars, like glucose, lactose and fructose, serve as energy sources for sperm (KATILA 1997). Examples of protectants include (milk and egg yolk) proteins and lipids as well as antioxidants.

Buffering compounds, like NaHCO₃ and 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethane sulfonic acid (HEPES), are typically added to semen extenders for maintaining the pH constant. The optimum pH for sperm function is described to be 6.5–7.6. In addition to maintaining sperm samples at this optimum pH, the medium osmolality should be ~300 mOsm kg⁻¹ (PICKETT et al. 1976). Sperm maintains equilibrium between the intra- and extracellular environment, via ionic exchange and water transport through the plasma membrane (i.e. via both active and passive transport). The medium osmotic pressure can be adjusted, e.g. via adding ions, sugars, and polyols. Ions like NaCl (and KCl) can be substituted in semen extenders
**Table 2.1** Selection of common used fresh semen diluents for stallion (INRA-82 (PALMER 1984), INRA-96 (MAGISTRINI et al. 1992), Kenney-extender (KENNEY et al. 1975)) and boar sperm (BTS (PURSEL and JOHNSON 1975)).

<table>
<thead>
<tr>
<th>fresh semen extender:</th>
<th>final concentration</th>
<th>INRA-82</th>
<th>INRA-96</th>
<th>Kenney-extender</th>
<th>BTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td>mg mL⁻¹</td>
<td>0.35</td>
<td></td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃ 8.4%</td>
<td>mL mL⁻¹</td>
<td></td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>mg mL⁻¹</td>
<td></td>
<td>0.118</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>mg mL⁻¹</td>
<td>9.52</td>
<td>4.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tri-sodium-dihydrate</td>
<td>mg mL⁻¹</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sodium citrate</td>
<td>mg mL⁻¹</td>
<td></td>
<td></td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>potassium citrate</td>
<td>mg mL⁻¹</td>
<td>0.82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>mg mL⁻¹</td>
<td></td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>mg mL⁻¹</td>
<td></td>
<td>1.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>mg mL⁻¹</td>
<td></td>
<td>0.4</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>mg mL⁻¹</td>
<td></td>
<td>0.14</td>
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<td></td>
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<tr>
<td>MgSO₄</td>
<td>mg mL⁻¹</td>
<td></td>
<td>0.2</td>
<td></td>
<td></td>
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<tr>
<td>glucose</td>
<td>mg mL⁻¹</td>
<td>50</td>
<td>13.21</td>
<td>49</td>
<td>37</td>
</tr>
<tr>
<td>lactose</td>
<td>mg mL⁻¹</td>
<td>3</td>
<td>45.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>raffinose</td>
<td>mg mL⁻¹</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>mg mL⁻¹</td>
<td></td>
<td></td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>UHT skim milk</td>
<td>mg mL⁻¹</td>
<td>1000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>native phosphor-caseinate</td>
<td>mg mL⁻¹</td>
<td></td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>skim milk powder</td>
<td>mg mL⁻¹</td>
<td></td>
<td></td>
<td>24</td>
<td></td>
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<tr>
<td>antibiotics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gentamicin</td>
<td>mg mL⁻¹</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
to maintain osmotic pressure. In addition to sugars like glucose, amino acids (e.g. histidine, carnitine) can serve as energy sources for sperm. In stallion sperm, the presence of deaminases and their role in amino acid metabolism has been described (SWEGEN et al. 2015).

2.2 Sperm diluents: protectants

Milk and egg yolk based semen extenders have proven especially beneficial in protecting sperm against the negative effects of cooled storage. Milk and milk-based products mainly consist of lipoproteins and phospholipids, while casein and lactoferrin are the main protein fractions. Milk compounds have been described to protect sperm via anti-oxidative action and preventing membrane lipid structural changes (MANJUNATH et al. 2002). There has been paid considerable effort in finding possibilities for replacing compounds of animal origin by defined compounds. Milk, for example, has been successfully replaced by native phosphocaseinate. Effective milk fractions can be obtained via microfiltration and ultrafiltration followed by freeze-drying of individual fractions (PIERRE et al. 1992). Several milk fractions and components have been tested for their abilities to preserve the motility and fertility of equine spermatozoa (BATELLIER et al. 1997).

Antioxidants are substances that delay, prevent or remove oxidative damage to biomolecules (i.e. lipids, proteins, and nucleic acids). They do so via enzymatic or scavenging activity (KHLEBNIKOV et al. 2007); via reacting with/inhibiting free radical oxidation reactions, by counteracting the auto-oxidation chain reaction, functioning as reducing agents or metal chelators as well as via inhibiting pro-
oxidative enzymes (CHOE and MIN 2006; LEOPOLDINI et al. 2006; PORKORNÝ 2007). Anti-oxidative activity is present in the equine seminal plasma but is removed when diluted semen is centrifuged. Antioxidants, however, can be added to extenders used for storing sperm samples. Examples of natural antioxidants are glutathione peroxidase, superoxide dismutase, and catalase, whereas coenzyme Q10 and L-carnitine are examples of non-enzymatic antioxidants (BOX et al. 1995; BRIGELIUS-FLOHE 1999; MRUK et al. 2002).

To protect sperm, extenders also often contain protective substances like bovine serum albumin (BSA) or polyvinyl alcohol (PVA). They have scavenger properties, which may prevent sperm membrane damage. Also, egg yolk, lecithin and low-density lipoproteins (LDL) have scavenger properties. Moreover, such compounds have been described to interact with the sperm membrane facilitating the protection and reducing cold-shock induced membrane changes (AMANN and PICKETT 1987).

Supplementing extenders with compounds of animal origin is associated with biosecurity risks. Also, semen itself may contain a specific microbiological load. Semen extenders are therefore supplemented with compounds that counteract microbiological growth, especially during longer storage at elevated temperatures. Antibiotics are added to counteract bacterial growth, whereas antimycotics are added to minimize fungal growth. Antibiotics typically added to semen extenders include members of the aminoglycoside (e.g. amikacin, gentamicin, streptomycin) and β-lactam (e.g. penicillin, ticarcillin) antibiotics.
Sperm of most mammalian species depends on glycolysis for energy production (STOREY 2008). Energy is needed for sperm movement, which is facilitated by the sperm flagellum. The enzymes and reactions involved in this process are located in the principal piece of the sperm tail. For glycolysis to take place, glucose needs to be taken up into the cytoplasm. Sugar can be transported through passive as well as active protein-mediated transport (BUCCI et al. 2010). Transmembrane transport proteins for glucose facilitate the transport of polar glucose molecules through the apolar membrane lipid bilayer. The following three subclasses of glucose transporters (i.e. GLUT protein family) have been described: (i) those which exhibit a particular tissue distribution, (ii) fructose-related transporters, and (iii) GLUT proteins that are translocated to the glycosylation site (SCHEEPERS et al. 2004). In stallion sperm, four members of the GLUT protein family have been characterized, including proteins belonging to classes I and II (GLUT1–3 and GLUT5, respectively).

In figure 2.1 a schematic presentation is shown illustrating how one glucose molecule (C₆H₁₂O₆) is converted into two pyruvate molecules via glycolysis (marked ‘1’). Similarly as glucose, also fructose can be converted via glycolysis. First glucose or fructose is converted into fructose-1,6-bisphosphate. This process is accompanied by the formation of two molecules dihydroxyacetone phosphate (DHAP) or glycerol aldehyde-3-phosphate (GAP). GAP can be directly used for further metabolic reactions, while DHAP first needs to be converted into GAP. During glycolysis, which compromises several metabolic reactions (i.e. 10 steps/reactions), eventually, one
glucose molecule gives rise to two pyruvate molecules, initially at the expense of two ATP molecules while later four ATP and two NADH molecules are produced. Pyruvate, in turn, can serve as a substrate for the citric acid cycle. The latter is also referred to as the tricarboxylic acid (TCA) cycle or Krebs cycle.

For the citric acid cycle, pyruvate originating from the glycolysis pathway can be used, or alternatively, pyruvate can be added to the medium. In all cases, pyruvate needs to be transported into the mitochondria where the citric acid cycle takes place and converted to acetyl-CoA through oxidative decarboxylation. In the citric acid cycle (marked ‘2’ in figure 2.1), acetyl-CoA is converted into citrate. Further reactions include isomerization, oxidation, and decarboxylation, eventually resulting in the formation of NADH, H⁺, and FADH₂ which serve as electron donors. Consequently, a proton-motive force directing out of the mitochondrial matrix can be generated, which is required for the production of ATP (see section 2.5).

2.4 Sperm metabolic pathways: Fatty acid oxidation

With the oxidation of fatty acids (FAO), fatty acids are converted to acetyl-CoA. This takes place in the mitochondrial matrix space (marked ‘3’ in figure 2.1). In this process, fatty acids can be used for energy production, since generated acetyl-CoA can enter the citric acid cycle. This results in the formation of NADH, H⁺, FADH₂ and eventually ATP (see above). For FAO, the first ATP needs to be converted into AMP and pyrophosphate. Initial, AMP can react with fatty acids, resulting in an acyl-adenylate formation. The separation of AMP from acyl-adenylate coincides with an energy release, which is necessary for the esterification of the acyl-group with
Figure 2.1 Overview of metabolic pathways for energy production. Glycolysis and amino acid metabolism take place in the cytosol, whereas the other pathways take place in the mitochondria. During glycolysis pyruvate is generated from glucose or fructose, which is transported through the membrane lipid bilayer into the mitochondria. Inside the mitochondria, it is converted to acetyl-CoA which is used as input for the citric acid cycle. Acetyl-CoA can also originate from fatty acid oxidation or amino acid catabolism. As a result, NADH, H⁺, and FADH₂ are formed. Via OXPHOS the protons are translocated to the intermembrane space, resulting in an electrochemical gradient. This gradient is the driving force for ATP synthase (as part of OXPHOS) to convert ADP and Pᵢ into ATP.
coenzyme A. The acyl group from produced acyl-CoA, in turn, can be transferred to carnitine which acts as a shuttle to transport the acyl-group across the membrane. Acyl-carnitine transported into the mitochondria can be split again and the acyl-group can be transferred to free coenzyme A, resulting in acyl-CoA. Subsequently, hydration and oxidation result in the conversion of NAD$^+$ to NADH and H$^+$ and FAD to FADH$_2$. The result is a shortening of fatty acids by two carbon atoms. Both carbon atoms are coupled to coenzyme A, resulting in the formation of acetyl-CoA as a final step of FAO. The process of fatty acid oxidation is proceeding until only two carbon units of the fatty acid remain.

2.5 Sperm metabolic pathways: Oxidative phosphorylation

The stallion sperm metabolism and energy generation have been described to be heavily reliant on oxidative phosphorylation (VARNER et al. 2015; PLAZA-DAVILA et al. 2016). Oxidative phosphorylation (OXPHOS) is an electron transfer chain driven by substrate oxidation (i.e. via citric acid cycle or FAO) that is coupled to ATP synthesis driven by an electrochemical gradient. OXPHOS occurs in the mitochondria located in the sperm midpiece and involves five protein complexes and 2 electron carriers (marked ‘4’ in figure 2.1). Electrons produced by the citric acid cycle are transferred through mitochondrial respiratory complexes I–IV, which results in proton translocation across the inner membrane and an electrochemical gradient between the inner mitochondrial membrane and the intermembrane space. This gradient, together with the action of ATP synthase (complex V) results in ATP synthesis. Proton transport via oxidation of NADH to NAD$^+$ can be performed directly
in complex I, whereas complex II is needed for oxidation of succinate to fumarate resulting in the transfer of electrons on FAD. Produced FADH$_2$ transfers its protons to complex III and IV. In complex III, a diversion from a two-electron transporter (ubiquinol) to a one-electron transporter (cytochrome c) takes place. In complex IV cytochrome c is oxidized. The released energy is used to pump protons from the matrix space into the intermembrane space. The proton driving force out of mitochondrial matrix results in a backflow of protons through the inner mitochondrial membrane, resulting in ATP production from ADP and inorganic phosphate (P$_i$) by ATP synthase.

2.6 Sperm metabolic pathways: Protein and amino acid catabolism

In addition to the sperm metabolic pathways described above, proteins and amino acids can be catabolized and be sources for energy production. Substrates for this alternative pathway are free amino acids and products of proteolysis. With the protein and amino acid catabolism (marked ‘5’ in figure 2.1), the carboxyl and amino groups are cleaved off and further utilized in the carbohydrate or lipid metabolism as well as in the citric acid cycle. The presence and function of enzymes needed for such utilization of proteins/amino acids have been shown for the bull as well as stallion sperm (TOSIC and WALTON 1950; SWEGEN et al. 2015).

Amino groups can be cleaved off through transamination and deamination reactions. With transamination, leading to transfer of the amino group (NH$_2$) of an amino acid to an alpha-keto acid, a new alpha-keto acid, and amino acid are formed. With deamination, amino acids are released as ammonia (NH$_3$) or ammonium ions.
(NH$_4^+$). Removing the carboxyl instead of the amino group, via decarboxylation, results in the formation of biogenic amines and the release of CO$_2$. The remaining carbon skeleton can be introduced to energy metabolism. Depending on how amino acids are cleaved from the carbon skeleton, they can be assigned to three different types: (i) glucogenic amino acids, which are degraded to pyruvate, (ii) ketogenic amino acids, which are converted to acetyl-CoA and (iii) mixed glucogenic/ketogenic amino acids that are degraded to acetyl-CoA. The acetyl groups are introduced to the citric acid cycle and support energy production.

2.7 Factors affecting storage stability of sperm: (Sodium) ion-exchange

Besides their essential function for viability, ions like Na$^+$, K$^+$, and Cl$^-$ can be transported across the cellular membrane for maintaining homeostasis. In addition to (the more rapid) movement of water, this is important for maintaining the cellular osmotic balance. Cell volume regulation and changes can be caused by (i) the attraction of macromolecules to each other at high concentrations (macromolecular crowding), (ii) changes in the concentration of specific ions or (iii) changes in the lipid bilayer (Hoffmann et al. 2009). In addition to volume changes due to water transport, cell volume changes are mediated by the activity of ion and volume sensitive transporters. Examples include Na$^+$/H$^+$ exchangers, Na$^+$-K$^+$-2Cl$^-$ cotransporters, and Na$^+$ channels.

The Na$^+$/H$^+$ exchange is mediated by the so-called Na$^+$/H$^+$ exchangers (NHE). In exchange for sodium ions, protons (H$^+$) actively migrate out of the intracellular
space. This is of importance for homeostasis, pH regulation, volume, and ion transport (DEMAUREX and GRINSTEIN 1994). In sperm, specific NHE isoforms (sNHE) are located in the principal piece of the flagellum (D. WANG et al. 2007). Under normal conditions, small variations in sodium concentration do not affect the function of Na⁺/H⁺ exchangers (MOOLENAAR et al. 1983). It has been found that exposure to hypertonic conditions results in NHE activation, whereas exposure to hypotonic condition has an inhibitory effect (KAPUS et al. 1994). Besides the extracellular sodium concentration, also the ATP content affects NHE activity. Although ATP is not required as driving force NHE, depletion of intracellular ATP causes a decreased Na⁺/H⁺ exchange due to modulation of proton-dependent regulatory mechanisms. This results in lowering the proton binding affinity of the exchanger by modulation of the proton-sensitive regulatory mechanism (CASSEL et al. 1986).

The Na⁺-K⁺-2Cl⁻ cotransporters (NKCC) belong to the cation-chloride cotransporter (CCC) superfamily. They are electron-neutral transport proteins that transport sodium, potassium and chloride ions across the cellular membrane. In addition to NKCC1 (CCC1) and NKCC2 (CCC2), this family also includes the Na⁺/Cl⁻ co-transporter NCC (CCC3) and the K⁺/Cl⁻ co-transporter KCC1, 2, 3 and 4. NKCC1 plays an essential role in the cell volume regulation and ion transport in the secretory epithelial tissue and can be detected in various organs of the reproductive system (DELPIRE et al. 1994). An NKCC1 deficiency leads to infertility due to erroneous spermatogenesis which is evident as a low number or complete absence of spermatozoa (PACE et al. 2000).
Ion exchange can also occur via transmembrane proteins. Sodium channels are ion channels of the cell membrane that have a specific conductivity for sodium ions. They have a pore structure surrounded by negatively charged amino acid residues whose arrangement makes the channels selective for positively charged sodium ions while repelling negatively charged ions. Increased intracellular sodium concentrations result in the activation of Na⁺-K⁺-ATPases to maintaining homeostasis. Na⁺-K⁺-ATPases can transport sodium ions out of the cell against the electrochemical gradient under ATP consumption. In case intracellular sodium levels are increased, this results in decreased sperm motility (TORRES-FLORES et al. 2011).

2.8 Factors affecting storage stability of sperm: Energy sources

Sugars, like glucose or fructose, provides the substrate for glycolysis whereas pyruvate and lactate represent essential sources for mitochondrial energy production. Pyruvate is produced during glycolysis from glucose (see section 2.3). Also, instead of pyruvate, lactate can be generated. This occurs, in equine spermatozoa, under anaerobic conditions by the action of lactate dehydrogenase C (LDH-C; SWEGEN et al. (2015)). Via the reverse reaction, LDH-C can convert lactate to pyruvate, which can be used as a substrate for oxidation to generate ATP via OXPHOS. Produced ATP, however, provides energy for motility and fertility. Pyruvate and lactate have been described to affect motility characteristics, in a dose-dependent manner (DARR et al. 2016). The metabolic flexibility of sperm allows the generation of glucose from OXPHOS too (see section 2.5).
Carnitine plays a role in energy production by supporting the β-oxidation of fatty acids. Also, carnitine can function as an antioxidant, scavenger and nonionic osmolyte. During β-oxidation, carnitine plays a role in transporting long-chain fatty acids from the cytosolic compartment to the matrix of mitochondria (see section 2.4) which in turn can be further metabolized for the citric cycle (STEIBER et al. 2004). In mammals, carnitine is produced in hepatocytes, transported by blood flow and secreted into the epididymal lumen (BROOKS 1980). The concentration of carnitine in seminal plasma has been described to correlate with sperm concentration, motility, and fertility (BORNMAN et al. 1989; SHEIKH et al. 2007). If added to diluents used for semen preservation, L-carnitine was found to result in reduced levels of sperm membrane lipid peroxidation and DNA damage (GIBB et al. 2015).

Also, adding rosiglitazone to semen extenders has been described to have beneficial effects in terms of improving sperm motility and viability. Rosiglitazone is a member of the class of thiazolidinedione proteins. These proteins rely on two different pathways for activation: (i) activation of peroxisome proliferator-activation receptor gamma (PPARG) and the (ii) non-PPARG pathway. PPARG is a nuclear receptor that functions in response to an increase in cellular glucose uptake (LEHMANN et al. 1995; DESVERGNE and WAHLI 1999).

Stallion sperm rely on the non-PPARG pathway (SWEGEN et al. 2016), which is initiated via AMP-activated protein kinase (AMPK). Rosiglitazone has a function in regulating metabolic flexibility and glucose uptake in various cell types (HALLSTEN et al. 2002), in response to detecting glucose levels. By regulating energy metabolism, rosiglitazone has been described to improve the quality of preserved sperm and protected the mitochondrial membrane potential. Through enhancing
glycolysis, it reduces the formation/accumulation of mitochondrial reactive oxygen species (N. WANG et al. 2019).

In stallion sperm, activation of AMPK has been described to result in a metabolic shift from consumption towards the production of ATP because of activation of glycolysis and β-oxidation (FRYER et al. 2002; SWEGEN et al. 2016).

### 2.9 Factors affecting storage stability of sperm: Reactive oxygen species (ROS) and oxidative stress

Reactive oxygen species (ROS) are produced in cells during normal metabolism. ROS like O$_2^-$ or H$_2$O$_2$ function as important second messengers via selective oxidation of molecules, including receptors, enzymes and/or transcription factors. Uncontrolled production of large amounts of ROS, however, may lead to sperm damage. Peroxidative damage to sperm membranes and DNA has been associated with male sub/infertility (AITKEN and BAKER 2006).

During spermatogenesis, a large percentage of the sperm cytoplasm is lost as well as enzymes such as catalase which normally can counteract oxidative stress. Especially the sperm membrane, which is rich in unsaturated fatty acids, is highly susceptible to peroxidative damage (JONES and MANN 1973). Negative effects of ROS on spermatozoa (i.e. in case of producing amounts that cannot be scavenged) include increased numbers of sperm with abnormal sperm morphology and membrane damaged sperm. Decreased sperm motility and induction of sperm DNA damage may eventually affect embryonic development (BALL et al. 2001; BAUMBER et al. 2003; LEWIS and AITKEN 2005).
As indicated above, the sperm have a limited amount of ROS scavengers. The equine seminal plasma contains compounds that have ROS scavenging activity; including catalase, superoxide dismutase, and glutathione peroxidase. A significant variation between stallions in the activity of catalase and superoxide dismutase has been reported (BALL et al. 2000; BAUMBER and BALL 2005). In addition to the enzymes mentioned above, seminal plasma contains substances that have similar functions. These include \( \alpha \)-tocopherol, ascorbic acid, glutathione, pyruvate, taurine, hypotaurine and albumin (ALVAREZ and STOREY 1983; HALLIWELL and GUTTERIDGE 1999; DE LAMIRANDE and GAGNON 1993).

Under physiological conditions, the membrane-associated NADPH oxidase 5 (NOX5) has been described to play a role in ROS production.

### 2.10 Factors affecting storage stability of sperm: Microbial load of fresh semen

Bacterial growth in freshly diluted semen is typically reduced by storage at cool temperatures (i.e. ~5°C) and the addition of antibiotics. Microorganism originating from the penis, prepuce, urethral fossa and environment can easily contaminate the ejaculate (VARNER et al. 1998). This is also true for healthy individuals. Commonly detected bacteria are Streptococcus dysgalactiae and equi subspecies (spp.), Bacillus ssp., Staphylococcus aureus, Escherichia coli, Pseudomonas spp. and Klebsiella spp. Also, molds and yeasts are commonly detected (JUAN and AHMED 2006; CORONA and CHERCHI 2009). Semen diluents that include sugars like glucose also serve as a growth medium for molds, yeasts, and bacteria; causing increased numbers during storage. Although not highly
pathogenic, such bacteria can cause endometritis when deposited in the female reproductive tract (SAMPER and TIBARY 2006). If sperm samples contain a high microbial content this typically leads to a decrease in the number of membrane intact sperm and also negatively affects sperm motility characteristics (ORTEGA-FERRUSOLA et al. 2009). This has been correlated with bacteria producing ROS (GORGA et al. 2001).

Typically combinations of antibiotics are added to semen extenders. Reports on effective combinations and concentrations, however, describe variable results. Also, here should be noted that some bacteria have developed resistance to specific antibiotics, which makes them ineffective (KILBURN et al. 2013; FRIERI et al. 2017). Antibiotics can be assigned to different groups. Here should be highlighted the following two groups, namely the aminoglycoside (e.g. amikacin, gentamicin, streptomycin) and β-lactam (e.g. penicillin, ticarcillin) antibiotics. Aminoglycosides are a heterogeneous group of oligosaccharide antibiotics in which two or more sugar or amino sugar molecules are linked via glycosidic bonds. They can bind to bacterial ribosomes resulting in the misreading of bacterial RNA. Beta-lactam antibiotics, on the other hand, function via inhibiting bacterial cell wall synthesis.

2.11 Effects of storage temperature on sperm viability

If lowering the temperature from room temperature down to 5°C spermatozoa exhibit a reduced metabolism (KATILA 1997). The membrane of sperm consists of a bilayer of phospholipids that undergo (thermotropic) membrane phase changes and domain formation known as ‘rafts’ (BROWN and LONDON 2000). Passing the
temperature range at which sperm undergo a phase transition, between 19–8°C, has been described to be especially harmful and has been associated with membrane leakiness and decreased sperm viability (DROBNIS et al. 1993; SIEME et al. 2015).

The lipid cholesterol content concerning the phospholipid content has been associated with resistance to cold-shock (POULOS et al. 1973; DARIN-BENNETT and WHITE 1977). Sperm exposed to cold-shock typically exhibit decreased motility and membrane intactness. Slow cooling rates <0.3°C per minute from 20–5°C are typically employed for cooling to limit the effects of cold-shock (PROVINCE et al. 1985; VARNER et al. 1988; KAYSER et al. 1992).

2.12 Methods for evaluation of sperm quality

The sperm concentration, percentages of motile and morphologically (ab)normal sperm have been described to play a role in determining male fertility and pregnancy rates in mares (SIEME et al. 2004; KARESKOSKI et al. 2019). Upon collection, these semen characteristics can be relatively easily documented. In addition to such assessments, however, more detailed analysis methods can be performed in which functional sperm characteristics are evaluated. In addition to the analysis of single sperm characteristics, the so-called ‘sperm cluster analyses’ can be performed. In the latter case, multiple characteristics are analyzed in parallel and sperm are categorized into various subpopulations.

Sperm motility is a good first parameter for assessing sperm viability and can be used to some extent for evaluating stallion fertility. Moreover, sperm motility and morphology do correlate and higher percentages of morphologically normal
spermatozoa are typically associated with higher percentages of motile sperm (LOVE 2011). Computer-assisted sperm analysis (CASA) refers to ‘standardized’ microscopic evaluation of sperm motility parameters. It should be noted, however, that there are no 'standard' thresholds and results may vary amongst laboratories. Besides the examination of percentages of progressive motile, local motile and immotile sperm, velocity parameters like the curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) can be analyzed. Other kinematic values that can be derived via this method include sperm movement of lateral head displacement (ALH) and beat-cross frequency (BCF) values (MORTIMER 1997, 2000).

Flow cytometric analysis of sperm stained with specific dyes (after exposure to specific conditions) allows for assessment of e.g. plasma and acrosomal membrane intactness, the mitochondrial membrane potential, as well as oxidized products. There are reports in which is postulated that determining sperm mitochondrial membrane potential characteristics is especially relevant for foreseeing sperm fertility. The mitochondrial membrane potential (MMP) refers electrochemical gradient that is formed during oxidative phosphorylation when protons are transported from the mitochondrial matrix. This electrochemical gradient is essential for ATP synthesis. Information about the MMP, mitochondrial function, and sperm energy status can be gained by using flow cytometry and the lipophilic cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide). This dye exhibits a color change as the mitochondrial membrane becomes more polarized (COSSARIZZA et al. 1993).
Increased production of superoxide anions can be evaluated via analyzing sperm membrane intactness and/or DNA damage. Membrane intactness is typically analyzed using membrane exclusion dyes like propidium iodide (PI). Such dyes cannot pass intact membranes but will exhibit fluorescence upon entering the cell in case of damaged membranes. In combination with Hoechst 33342 or SYBR-14, sperm with intact membranes can be stained and differentiated. For detecting membrane lipid peroxidation BODIPY is available.

### 2.13 Methods for determining sperm chromatin intactness and capacitation

As mentioned before, increased production of superoxide anions can lead to DNA damage. Male fertility, sperm chromatin structure/integrity, and DNA damage can be analyzed using the sperm chromatin structure assay (SCSA). With this assay, the susceptibility of sperm DNA to acid denaturation is analyzed (EVENSON et al. 1980). After treating sperm with acid and staining with acridine orange, the so-called DNA fragmentation index (DFI) can be derived. DFI values are highly correlated with stallion sperm fertility (LOVE and KENNEY 1998).

Stallion fertility can also be analyzed by evaluating the ability of spermatozoa to undergo capacitation in response to specific treatments. Capacitation refers to the process that sperm undergo after ejaculation in the female genital tract for preparing the fertilization of an oocyte. This process involves several reactions, including calcium influx. Fluorescent dyes like Fluo-3/AM can be used to determine the intracellular calcium content of the sperm. Sperm binding to the zona pellucida of oocytes is an important step in fertilization.
The fertility of stallions with unknown fertility potential actually can be analyzed via a so-called hemi-zona assay in which sperm binding (to one half of an oocyte) is directly compared with the binding capacity of a stallion of proven fertility (FAZELI et al. 1995).
3 MATERIALS AND METHODS

3.1. Semen collection and processing

Sperm samples used for the experiments described in this study were aliquots taken from ejaculates that were collected as part of the commercial artificial insemination program of the State Stud of Lower Saxony, Celle, Germany. Semen was collected three times a week, from warmblood stallions of the Oldenburg and Hanoverian breed (7−21 years). Stallions were held and semen collections were performed according to national and European regulations and use protocols. Stallions were kept in box stalls bedded with wooden shavings or straw. They were fed three times a day with grain, mineral feed, and hay, and had ad libitum access to water. All stallions were healthy and in good physical condition. Semen was collected using a breeding phantom and an artificial vagina (model ‘Celle’ and ‘Hanover’, respectively; Minitüb, Tiefenbach, Germany) in the presence of a teaser mare. The artificial vagina was used with a single-use inner plastic liner (Minitüb, Tiefenbach, Germany) and semen was collected in an attached sterile glass bottle, which was pre-warmed at 38°C. Sterile milk filter (Eimermacher, Nordwalde, Germany) was used to remove the gel fraction and dirt. The artificial vagina was filled with 41−43°C warm water and sterile petroleum jelly was added as a lubricant on the inner liner.

Directly after semen collection, the sperm concentration was determined using a photometer (SMD1; Minitüb, Tiefenbach, Germany), after which semen was divided into aliquots and diluted to 100×10⁶ sperm mL⁻¹ with pre-warmed diluent of 38°C. As diluent, INRA-96 (IMV technologies, L’Aigle, France) was used or modified equine
Biggers, Whitten and Whittingham Medium (m-eBWWM) as described in detail below (see section 3.2). Diluted semen was centrifuged at 600×g for 10 min, after which the supernatant was removed. The pellet was resuspended in the remaining diluent (i.e. an approximate similar volume in all aliquots), the sperm concentration was determined using a counting chamber (Thoma ‘neu’; Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim v. d. Rhön, Germany), after which sperm was diluted with fresh diluent (i.e. INRA-96 or m-eBWWM) to 50×10^6 sperm mL⁻¹ for storage at 5°C and higher temperatures.

3.2. Preparation of modified equine Biggers, Whitten and Whittingham Medium: m-eBWWM, with varying NaCl, ChCl, L-histidine and L-carnitine contents

For storing stallion sperm samples at 5°C as well as higher temperatures, diluents were tested that were modifications of Biggers, Whitten and Whittingham Medium (BWWM; WHITTEN and BIGGERS (1968)). Modified equine BWWM (m-eBWWM) as described by GIBB et al. (2015) is composed of: 95 mM NaCl, 4.7 mM KCl, 1.7 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 20 mM HEPES, 5.6 mM D-glucose, 275 μM sodium pyruvate, 3.7 μL mL⁻¹ 60% sodium lactate syrup (i.e. 20 mM sodium lactate), 50 U mL⁻¹ penicillin, 50 μg mL⁻¹ streptomycin, 250 μg mL⁻¹ gentamicin. Furthermore, 0.1% (w/v) polyvinyl alcohol (PVA) is added. In later versions, m-eBWWM was modified (GIBB et al. 2016) and NaCl was replaced by ChCl (95 mM) or L-histidine alone (200 mM) or L-histidine in combination with L-carnitine (100 mM each).

The modified eBWW media that were tested in this study we refer to as m-
eBWWM-1−7. These were prepared using single component stock solutions, which were prepared as listed in table 3.1. Na-lactate syrup was added as purchased, while L-histidine and antibiotics were added as powders in the final solutions.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. In table 3.2 compositions (i.e. final concentrations) are listed of the m-eBWWM-1−7 solutions. All media had a pH 7.4±0.02 and osmolality of ~295−355 mOsm kg−1, dependent on the formulation, whereas the addition of skim milk powder resulted in osmolalities up to 500 mOsm kg−1. In experiment 1 and 2, m-eBWWM formulations were tested in which NaCl was replaced (in part) by ChCl (m-eBWWM-1−3; 95/0, 47.5/47.5, 0/95 mM NaCl/ChCl) or by L-histidine alone or in combination with L-carnitine (m-eBWWM-4−5; 200/0, 100/100 mM L-histidine/L-carnitine). Also, modifications of m-eBWWM-5 were tested which included supplementation with up to 5.0% (w/v) skim milk powder (SMP; m-eBWWM-5a−e). The formulation supplemented with 0.5% SMP was selected for further testing of supplements and is referred to as m-eBWWM-6. Supplements that were added to m-eBWWM-6 included 0.1% PVA and 75 μM coenzyme Q10 (Q10; m-eBWWM-7a and b).

3.3 Evaluation optimum osmolality of an m-eBWWM formulation containing skim milk powder

For evaluation of effects of the medium osmolality, m-eBWWM-6 containing 0.5% SMP of 300 mOsmol kg−1 was modified; to produce hypotonic and hypertonic variants.
### Table 3.1

Stock solutions used for preparing m-eBWWM. All were prepared as 500 mM solutions in water, except rosiglitazone which was solved in DMSO at 140 mM. These stock solutions can be stored for several months at 5°C.

<table>
<thead>
<tr>
<th>m-eBWWM stock solutions</th>
<th>prepared by dissolving (g per mL)</th>
<th>molecular weight (g per mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>final concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 mM CaCl₂</td>
<td>36.76 g 500 mL water</td>
<td>MW (CaCl₂×2H₂O) 147.02 g mol⁻¹</td>
</tr>
<tr>
<td>500 mM ChCl</td>
<td>34.91 g 500 mL water</td>
<td>MW (ChCl) 139.62 g mol⁻¹</td>
</tr>
<tr>
<td>500 mM D-glucose</td>
<td>49.54 g 500 mL water</td>
<td>MW (D-glucose×1H₂O) 198.17 g mol⁻¹</td>
</tr>
<tr>
<td>500 mM HEPES</td>
<td>59.58 g 500 mL water</td>
<td>MW (HEPES) 238.31 g mol⁻¹</td>
</tr>
<tr>
<td>500 mM KCl</td>
<td>18.64 g 500 mL water</td>
<td>MW (KCl) 74.55 g mol⁻¹</td>
</tr>
<tr>
<td>500 mM KH₂PO₄</td>
<td>34.02 g 500 mL water</td>
<td>MW (KH₂PO₄) 136.09 g mol⁻¹</td>
</tr>
<tr>
<td>500 mM L-carnitine</td>
<td>40.30 g 500 mL water</td>
<td>MW (L-carnitine) 161.20 g mol⁻¹</td>
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<tr>
<td>500 mM MgSO₄</td>
<td>61.62 g 500 mL water</td>
<td>MW (MgSO₄×7H₂O) 246.48 g mol⁻¹</td>
</tr>
<tr>
<td>500 mM NaCl</td>
<td>14.61 g 500 mL water</td>
<td>MW (NaCl) 58.44 g mol⁻¹</td>
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<tr>
<td>500 mM NaHCO₃</td>
<td>21.00 g 500 mL water</td>
<td>MW (NaHCO₃) 84.01 g mol⁻¹</td>
</tr>
<tr>
<td>500 mM Na-pyruvate</td>
<td>0.06 g 1 mL water</td>
<td>MW (Na-pyruvate) 110.04 g mol⁻¹</td>
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<tr>
<td>140 mM rosiglitazone</td>
<td>0.05 g 1 mL DMSO</td>
<td>MW (rosiglitazone) 357.43 g mol⁻¹</td>
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</table>
Table 3.2 Table summarizing the m-eBWWM formulations tested. Formulations 1–5 were used in 'Experiments 1 and 2'. In 'Experiment 3', formulation 5 was supplemented with 0.25−5.0% (w/v) skim milk (formulation 5a−e), whereas in 'Experiment 4' 0.5% (w/v) skim milk was supplemented with 0.75 mM Q10 or 0.1% (w/v) PVA (formulation 7). In 'Experiment 5−7', formulations of m-eBWWM-6 without antibiotics added were tested.

<table>
<thead>
<tr>
<th>final concentration</th>
<th>m-eBWWM formulation:</th>
<th>-1-</th>
<th>-2-</th>
<th>-3-</th>
<th>-4-</th>
<th>-5-</th>
<th>-6-</th>
<th>-7-</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ mM</td>
<td>4.70</td>
<td>4.70</td>
<td>4.70</td>
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<td>4.70</td>
<td>4.70</td>
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<tr>
<td>glucose mM</td>
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<td>5.60</td>
<td>5.60</td>
<td>5.60</td>
<td>5.60</td>
<td>5.60</td>
<td>5.60</td>
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</tr>
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<td>20.00</td>
<td>20.00</td>
<td>20.00</td>
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</tr>
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<td>KCl mM</td>
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<td>4.70</td>
<td>4.70</td>
<td>4.70</td>
<td>4.70</td>
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</tr>
<tr>
<td>KH₂PO₄ mM</td>
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<td>1.20</td>
<td>1.20</td>
<td>1.20</td>
<td>1.20</td>
<td>1.20</td>
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<tr>
<td>MgSO₄ mM</td>
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<td>1.20</td>
<td>1.20</td>
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</tr>
<tr>
<td>NaHCO₃ mM</td>
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<td>25.00</td>
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<td>pH</td>
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</table>
The osmolality was adjusted to 100, 200, 250, 350, 400 and 500 mOsmol kg$^{-1}$, via diluting with water or adding NaCl. Before exposure to anisotonic media, raw semen was diluted in INRA-96 or m-eBWWM-6 of 300 mOsmol kg$^{-1}$ and centrifuged at 600×g for 10 min, after which the supernatant was removed. Pre-warmed INRA-96 or m-eBWWM-6 of 38°C was added to the sperm pellet, for obtaining a 50×10$^6$ sperm mL$^{-1}$ sperm sample. This sample was cooled to room temperature and then stored for 2 h at 5°C. After storage, 1 mL sperm samples/aliquots were transferred into 1.5-mL microtubes for centrifugation (1000×g for 10 min), after which 950 μL of the supernatant was removed. After that, sperm were resuspended in 1 mL medium of a specific osmolality and sperm motility characteristics were evaluated after incubation for 10 min at 38°C.

### 3.4 Cooled storage of diluted semen, and storage at elevated temperatures

In the case of analyzing sperm characteristics during storage, diluted sperm was divided into aliquots for analysis at different time points. To minimize exposure to air (i.e. oxygen), sperm samples were stored under anaerobic conditions. This was done by fully filling 1.5-mL microtubes. After processing and dilution as described in section 3.1 and 3.9–13, sperm samples were stored at temperatures ranging from 5−30°C. Storage at 5°C was done in a commercial fridge, whereas storage at 17°C was done in an incubator commonly used for storing porcine semen (Minitüb, Tiefenbach, Germany). Room temperature storage was done in the lab in a polystyrene box, while the temperature profile was documented using a T-type thermocouple (Fluke, Everett, WA, USA). For storage at 30°C, an incubator was
used (Memmert GmbH, Schwabach, Germany).

3.5 Flow cytometric analysis of sperm membrane intactness and intracellular sodium content

Sperm stained with various fluorescent dyes were analyzed using a flow cytometer (CytoFLEX; Beckman-Coulter, Brea, CA, USA), equipped with three lasers and 13 filters. For the dyes used in the current studies (described below), the 488 nm blue laser (50 mW) was used for excitation and green, and red fluorescence/emission was detected via respectively the 525/40 and 585/42 nm bandpass filters (i.e. FITC and PC5.5 filters).

To determine sperm plasma and acrosomal membrane integrity samples were stained with both propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) and peanut agglutinin with linked fluorescein isothiocyanate (FITC-PNA; Axxora, San Diego, CA, USA). PI exhibits red fluorescence upon binding nuclear DNA in case of passing damaged plasma membranes, whereas FITC-PNA fluoresces green upon binding lectins inside membrane-damaged acrosomes. Unstained sperm are classified as membrane intact and viable, whereas sperm exhibiting green fluorescence are classified as acrosomal membrane damaged/acrosome-reacted.

For flow cytometric analysis, 20 μL sperm sample (50×10⁶ sperm mL⁻¹) was added to 475 μl HEPES buffered saline (HBS; 137 mM NaCl, 20 mM HEPES, 10 mM glucose, 2.5 mM KOH), supplemented with 2 μL 0.75 mM PI and 3 μL 0.75 μM FITC-PNA; resulting in 1×10⁶ sperm mL⁻¹, 3.0 μM PI and 0.45 μM FITC-PNA. Samples were incubated at room temperature for 10 min in darkness, after which 10000 sperm were
analyzed and selected based on their forward and side scatter properties, using a flow rate of 10−20 events per second. Percentages of plasma and acrosomal membrane intact and damaged sperm were derived from plots in which red fluorescence (FL3) versus green fluorescence (FL1) intensities were plotted.

In order to obtain insights in intracellular sodium contents, 1 μL 0.25 mM sodium green (SG; in 100% DMSO) was added to 499 μL 50×10⁶ sperm mL⁻¹ sperm sample (in diluent used for storage) resulting 5 μM SG (and 0.20% DMSO). Samples were incubated for 30 min at RT in darkness, after which 10 μL SG-stained sperm sample was added to 488 μL HBS, supplemented with 2 μL 0.75 mM PI (i.e. the final concentration of 1×10⁶ sperm mL⁻¹ and 3.0 μM PI) for flow cytometric measurements as described above. Green fluorescence intensities were determined as mean and median values, for all sperm as well as membrane intact and damaged sperm (PI-negative and -positive sperm).

3.6 Computer-assisted sperm analysis of motility characteristics

Sperm motility characteristics were analyzed using computer-assisted sperm analysis (CASA). The setup used included a microscope with a temperature-controlled stage and camera (Androvision; Minitüb, Tiefenbach, Germany). All materials used for CASA were pre-warmed to 38°C. Software settings were used that were provided by the manufacturer, as ‘species-specific basic settings’.

Prior to CASA analysis, 500 μL sperm samples (50×10⁶ sperm mL⁻¹; in diluent used for storage) were transferred into 1.5-mL tubes and then incubated for 5 min at 38°C. After gently mixing, 3 μL was taken and added into a chamber of a Leja 20
micron four-chamber slide (Leja Products BV, Nieuw Vennep, Netherlands) which was maintained at 38°C. Per sample, four microscopic fields (i.e. ~600 sperm) were analyzed, and percentages of (progressively) motile sperm were derived as well as other parameters describing motility characteristics [including the curvilinear velocity (VCL; µm), tail beat cross frequency (BCF; Hz), amplitude of the lateral head movement (ALH; µm), straight-line velocity (VSL; µm sec\(^{-1}\)), straightness (STR, \(\text{STR}=\text{VSL}/\text{VAP} \times 100\); %) and linearity (LIN, \(\text{LIN}=\text{VSL}/\text{VCL} \times 100\); %)]. Sperm were considered immotile when their average path velocity was lower than 20 µm \(\text{s}^{-1}\), and local motile in case of an average path velocity ranging from 20−40 µm \(\text{s}^{-1}\). Sperm were classified as progressively motile if they had an average path velocity higher than 40 µm \(\text{s}^{-1}\) and straightness movement greater than 0.5 (straight line versus average path velocity, VSL/VAP, in relative units). Hyperactive motility was characterized by analyzing the velocity parameter based on publications of RATHI et al. (2001) and MCPARTLIN et al. (2009).

### 3.7 Flow cytometric analysis of sperm chromatin structure and integrity

To evaluate sperm nuclear chromatin structure and integrity, the sperm chromatin structure assay (SCSA) was used (EVENSON et al. 1980). Therefore, samples were frozen via plunging directly in liquid nitrogen and stored in liquid nitrogen until analysis. Before analysis, samples were thawed in a 38°C water bath and diluted with TNE buffer (0.15 M NaCl, 0.01 M TRIS-HCl, 1 mM Na\(_2\)EDTA, pH 7.4) to ~2×10⁶ sperm mL\(^{-1}\). Then, 200 µL was transferred to a clean tube, and 400 µL acid solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2) was added,
after which the sample was vortexed for 30 seconds. Immediately after that, 1.2 mL acridine orange staining and neutralization solution (0.15 M NaCl, 0.037 M citric acid, 0.126 M Na₂HPO₄, 0.0011 M Na₂EDTA, pH 6.0; containing 6 μg mL⁻¹ acridine orange) was added, for staining single- and double-stranded DNA while stopping the denaturation reaction. Samples were stored on ice for 3 min, before analyzing using a FACScan flow cytometer (Becton-Dickinson, Heidelberg, Germany). This flow cytometer contained a 488 nm laser with three filters, from which the 530/30 nm bandpass and 650 nm long-pass filters were used for respectively detecting green and orange/red fluorescence (i.e. FL1 and FL3).

After challenging sperm chromatin with acid denaturation and adding acridine orange, DNA integrity can be examined by looking at the sperm populations exhibiting green and orange fluorescence in FL1 versus FL3 plots. Acridine orange exhibits green fluorescence upon binding double-stranded DNA, whereas it fluoresces orange in the case of binding to single-stranded DNA. DNA-fragmentation index (DFI) values were determined using DAS (BEISKER 1994) and Kaluza software (Beckman Coulter, Brea, CA, USA), via the method described by EVENSON et al. (2002). In short, in FL1 versus FL3 plots, first cellular debris (and events exhibiting high green fluorescence) is taken out from the analysis. Then, plots are made in which FL1 is plotted versus FL3/(FL1+FL3). From such plots, sperm numbers in the two apparent sperm populations can be derived, which in turn can be used to calculate the percentage of sperm outside the main population (COMPα₁) and DFI-value. LOVE et al. (2002) has described DFI-ranges that correlate with stallion fertility potential.
3.8 Assessment of bacterial growth

A commercially available kit (Easicult Combi; Orion Diagnostica, Hamburg, Germany) was used to detect bacteria and fungi in stored sperm samples, according to the instructions provided by the manufacturer. The test slides provided with the kit contain a different type of agar on each side, supporting respectively growth of most common bacteria and fungi. In short, for just wetting test slides with the sperm samples, 0.7 mL sperm sample was added onto a test slide and access solution was removed after 5–10 seconds by decanting. Thereafter, slides were transferred in the tubes provided with the kit which were closed and incubated at 30°C. Bacterial growth was inspected after 1–2 d incubation and fungal growth after 3 d. At the respective days, photos were taken and the number of colonies was determined per slide and expressed as colony-forming units (CFU) per square centimeter.

3.9 Experiment 1 and 2: Sperm viability during cooled storage in m-eBWW media with varying NaCl, ChCl, L-histidine and L-carnitine contents; with(out) initial dilution in INRA-96

In experiment 1, m-eBWWM-1–5 was used, both for diluting raw semen and resuspending sperm after centrifugation. In contrast, in experiment 2, raw semen was diluted in INRA-96 and sperm was resuspended in m-eBWWM-1–5 after centrifugation. In both cases, semen from six different stallions was used, and INRA-96 was used as a control (i.e. used both for initial dilution and resuspending after centrifugation). Figure 3.1 presents a schematic presentation of the study designs.
Figure 3.1 Experimental designs of ‘Experiment 1’ and ‘Experiment 2’. In ‘Experiment 1’, m-eBWWM-1−5 was used both for diluting raw semen and resuspending sperm after centrifugation. In contrast, in ‘Experiment 2’, raw semen was first diluted in INRA-96 and sperm was resuspended in m-eBWWM-1−5 after centrifugation. The m-eBWWM formulations tested differed with respect to their NaCl content which was replaced (in part) by ChCl, L-histidine and/or L-carnitine. Sperm samples were stored under anaerobic conditions at 5°C, for up to 7 d. Sperm membrane intactness and motility characteristics were determined during storage.
Semen was collected and prepared as described in detail above. Initial dilution was directly after collection, for transportation to the lab. In the lab, samples were subjected to centrifugation processing and final dilution, which was within 1–3 h after semen collection. For analysis at different time points, separate 1.5-mL tubes were prepared that were completely filled with sperm samples for storage under anaerobe conditions. Samples were stored at 5°C in a commercial fridge. Sperm characteristics were analyzed ~4 h after dilution in the medium used for storage, as well as after 1, 3 and 7 d storage. Sperm characteristics that were analyzed included motility characteristics as well as acrosomal and plasma membrane intactness and intracellular sodium contents.

3.10 Experiment 3: Sperm viability during cooled storage in m-eBWW media supplemented with skim milk powder (SMP)

For experiment 3, m-eBWWM-5 was supplemented with 0.25/0.5/1.56/2.5/5.0% SMP; which is referred to as m-eBWWM-5a–e. Later, m-eBWWM-5 supplemented with 0.5% SMP was referred to as m-eBWWM-6. The same media types were used both for diluting raw semen and resuspending sperm after centrifugation. Dilution in INRA-96 and m-eBWWM-6 not supplemented with SMP was performed for comparison. Also, the influence of PVA was tested in this experiment. Figure 3.2 presents a schematic presentation of the study design, in which can be seen that the collection and storage procedures were similar as described above for experiment 1 and 2. Motility, as well as acrosomal and plasma membrane intactness, were analyzed during storage at 5°C for up to 7 d.
Figure 3.2 Experimental design of ‘Experiment 3’. Therefore m-eBWWM-5 was used alone or by supplementing different concentrations of skim milk powder and/or 0.1 % (w/v) polyvinyl alcohol. INRA-96 functioned as reference. Each media was used both for diluting raw semen and resuspending sperm after centrifugation.
3.11 Experiment 4: Sperm viability during cooled storage in m-eBWWM supplemented with coenzyme Q10

In experiment 4, m-eBWWM-6 (i.e. m-eBWWM containing 100/100 mM L-histidine/L-carnitine and 0.5% SMP) was used to evaluate effects of also adding 75 µM coenzyme Q10. Care was taken that the initial medium osmolality was 300 mOsmol kg\(^{-1}\). Initial dilution of fresh semen was done in the same medium that was used for storage, and INRA-96 and m-eBWWM-6 without further supplements were used for comparison. Further processing and storage procedures were similar as described above for experiments 1 and 2; sperm was stored up to 7 d at 5 °C and sperm characteristics that were analyzed included motility characteristics and sperm membrane intactness.

3.12 Experiment 5 and 6: Sperm viability in m-eBWWM with and without antibiotics, if stored at elevated temperatures

For experiments 5 and 6, m-eBWWM-6 was prepared both without and with antibiotics added. If added, antibiotics were used at the following final concentrations: 50 U mL\(^{-1}\) penicillin, 250 U mL\(^{-1}\) gentamicin, 38 U mL\(^{-1}\) streptomycin and 100 U mL\(^{-1}\) nystatin. In experiment 5, m-eBWWM with/out antibiotics was used both for diluting fresh semen and resuspending sperm after centrifugation, whereas in experiment 6 INRA-96 was used for diluting fresh semen, followed by diluting the sperm pellet obtained after centrifugation in m-eBWWM (figure 3.3). Further processing was similar, as described above for experiments 1 and 2.
Figure 3.3 Experimental design of 'Experiment 5' and 'Experiment 6'. In 'Experiment 5', m-eBWWM-6 (m-eBWWM-5 containing 0.5% (w/v) skim milk powder) with and without antibiotics was used both for diluting raw semen and resuspending sperm after centrifugation, respectively. Sperm samples were stored under anaerobic conditions at storage temperatures from 5−30 °C, for up to 7 d. Sperm membrane intactness and motility characteristics were determined during storage. In contrast, in 'Experiment 6', raw semen was first diluted in INRA-96 and sperm was resuspended in m-eBWWM-6 after centrifugation.
Sperm was stored up to 7 d at different storage temperatures ranging from 5–30°C. Sperm characteristics that were analyzed included motility characteristics and sperm membrane intactness. Details on the various antibiotics used are listed in table 3.3.

Table 3.3 Table summarizing the antibiotics used for m-eBWWM and INRA-96 (recalculated; DEAN et al. (2012)). Formulations of m-eBWWM with and without antibiotics were used in ‘Experiments 5–7’.

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<th>INRA-96 commercial available</th>
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<td>Penicillin</td>
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<tr>
<td>amphotericin B</td>
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3.13 Experiment 7: Effect of using diluents with and without antibiotics for initial dilutions as well as storage of sperm samples

In experiment 7, m-eBWWM-6 formulations without and with antibiotics were prepared to evaluate the need for the presence of antibiotics for initial dilution of semen as well as storage of diluted sperm samples at 5°C and 17°C. Initial dilution (and storage) in INRA-96 was used for comparison. A similar procedure was followed as described above (see section 3.1). See table 3.3 for details concerning the used
antibiotics. For the first dilution INRA-96 or m-eBWWM-6 without or with antibiotics were used. After centrifugation, sperm were diluted in m-eBWWM-6 without antibiotics for all cases after which samples were stored under anaerobic conditions at 5°C or 17°C for 7 d. Sperm motility characteristics, membrane, and chromatin intactness were analyzed on d 0, d 3 and d 7.

In addition, bacterial and fungal growth was followed using Easicult Combi test slides. This was done exemplary using sperm samples from 3 different stallions, after 3 and 7 d storage at 5 as well as 17°C. Also, a microbiological analysis was done using raw semen from 6 different stallions directly after semen collection. In the case of analyzing diluted sperm, 50×10⁶ sperm mL⁻¹ in diluent was added per test slide, whereas raw semen was applied as obtained (sperm concentration ranging from 176–311 sperm mL⁻¹); in both cases, 0.7 mL was used.

3.14 Statistic analyses

The statistical evaluation was carried out using the 'SAS' software (SAS Institute Inc., Cary, NC, USA) in accordance with the instructions for use provided by the Institute for Biometry and Epidemiology of the University of Veterinary Medicine Hannover. Before analyzing, the data were checked for normal distribution to make a decision choosing parametric or non-parametric tests for data analyze. Using ‘ANOVA’ was chosen to analyze differences in sperm characteristics (i.e. membrane intactness, motility, DFI) between time points of analysis (i.e. directly after semen processing and storage durations up to 7d) as well as different diluents. Additionally, multiple comparisons of means were performed using Tukey’s test. Variances were
evaluated as statistically significant with a value $p<0.05$. The data were presented as mean values with the corresponding standard deviations.
4. RESULTS

4.1 Sperm motility and membrane intactness during cooled storage in m-eBWWM; with different NaCl, ChCl, L-histidine, and L-carnitine contents

It was tested if replacing NaCl by ChCl, L-histidine and/or L-carnitine in storage media for stallion sperm increased sperm viability during cooled storage. Therefore various m-eBWWM media were used, both for use with centrifugation and storage (figure 4.1) and storage only after centrifugation in INRA-96 (figure 4.2). In figure 4.1, it can be seen that partial or complete replacement of NaCl by ChCl resulted in a significant (p<0.05) increased percentage of membrane intact sperm. After 1 d storage at 5°C, percentages were respectively 22±15% and 27±15% whereas this was 9.0±5.7% in medium with NaCl. Membrane intactness was further increased in the case of replacement with L-histidine (40±17%; p<0.05). Percentages were significantly highest using a combination of L-histidine and L-carnitine (48±18%; p<0.05). The ratio of membrane intact sperm significantly decreased with longer storage durations, while the above-described differences amongst the media were also seen after 3 and 7 d storage (p<0.05). At all times, the highest numbers of membrane intact sperm were found if using m-eBWWM substituted with L-histidine and L-carnitine (42±18% and 34±17% at d 3 and 7, respectively). A similar trend was seen when looking at the percentages of motile sperm. The amount of motile sperm, however, decreased more rapidly. On the day of semen collection, percentages of motile sperm ranged from 54−64%. In the case of using m-eBWWM containing NaCl, the percentages of motile sperm decreased below 8.9±2.4% after 1 d storage. In case NaCl was replaced by ChCl or L-
histidine/L-carnitine, respectively 20±9.4 and 32±14% of sperm were motile. In the latter case, motility was 29±13% at d 3 and 19±8.2 % at d 7. Percentages of progressively motile sperm were numeric (1–24%) lower but showed similar trends. For comparison, sperm was also centrifuged and stored in INRA-96. Then, membrane intactness values remained around 77±2.2% during storage for up to one week. Sperm motility percentages only decreased to 61±8.8% at d 3 and 48±2.1% at d 7. The ratio of progressively motile sperm ranged from 43–35%.

Next, it was tested if initial dilution and centrifugation in INRA-96 prior to diluting with m-eBWWM for cooled storage increased sperm viability. On the day of semen collection, membrane intactness was found to be significantly higher as when using m-eBWWM for centrifugation (78–76% versus 40–75%; p<0.05). There was a rapid decrease in the percentage of membrane intact sperm within 1 d cooled storage, which was 64±11% if using L-histidine/L-carnitine, 51±11% if using ChCl, and only 20±9.4% if NaCl was used (p<0.05). The decrease continued (with a slower rate) until d 7, resulting in substantial differences between the different m-eBWWM formulations. In the case of using NaCl, 4.5±2.4% membrane intact sperm were found, whereas this was significantly (p<0.05) increased to 26±7.7% and 61±10% in case of substituting NaCl with respectively ChCl or L-histidine/L-carnitine. Sperm membrane intactness was higher with using INRA-96 as a first dilution as compared to using m-eBWWM with L-histidine/L-carnitine both as a primary and final diluent (61±10% versus 34±17% on d 7; p<0.05). Similar as described above, the differences seen with sperm membrane intactness were also observed when looking at motility.
Figure 4.1 Percentages of membrane intact (A,B) and motile (C,D: dark colors: total motility; light colors: progressively motile) sperm during storage for up to 7 d at 5°C in m-eBWWM-1–5 with varying NaCl, ChCl, L-histidine and L-carnitine contents (‘Experiment 1’; see methods section for detailed descriptions). Directly after collection, sperm were diluted in INRA-96 or m-eBWWM-1–5, after which samples were centrifuged and resuspended in the fresh medium of the same composition for cooled storage. The m-eBWW media contained NaCl/ChCl (mM) 95/0 (red symbols), 47.5/47.5 (red/blue symbols), 0/95 (blue symbols) or His/Car (mM) 200/0 (green symbols), 100/100 (green/yellow symbols) mM. For comparison, sperm were also stored in INRA-96 (orange symbols). Data are presented as line plots (A,C) as well as bar plots (B,D). Values marked with letters differ significantly (p<0.05) between diluents, whereas numbers differ significantly (p<0.05) between time point within a single diluent. Mean values ± standard deviations are presented, which were calculated from six ejaculates from six different stallions.
Figure 4.2 Percentages of membrane intact (A,B) and motile (C,D: dark colors: total motility; light colors: progressively motile) sperm during storage for up to 7 d at 5°C in m-eBWWM-1–5 with varying NaCl, ChCl, L-histidine and L-carnitine contents (‘Experiment 2’; see methods section for detailed descriptions). In contrast to ‘Experiment 1’, INRA-96 was used for all cases as the first diluent for fresh semen. After centrifugation, sperm were diluted in INRA-96 or m-eBWWM-1–5 and stored for 7 d at 5°C. During storage duration sperm characteristics were analyzed. The m-eBWWM media contained NaCl/ChCl (mM) 95/0 (red symbols), 47.5/47.5 (red/blue symbols), 0/95 (blue symbols) or His/Car (mM) 200/0 (green symbols), 100/100 (green/yellow symbols). In addition, sperm was stored in INRA-96 (orange symbols). Values marked with letters differ significantly (p<0.05) between diluents, whereas numbers differ significantly (p<0.05) between time point within a single diluent. Mean values ± standard deviations are presented, which were calculated from six ejaculates from six different stallions.
Replacing NaCl by ChCl alone or with a combination of L-histidine and L-carnitine resulted in significantly (p<0.05) higher motility. Percentages of total motile sperm ranged from 58–68% at d 0.

During storage motility decreased significantly (p<0.05) with different rates dependent on the formulation used. At d 7, motility values were numeric six-fold higher in the case of m-eBWWM with L-histidine and L-carnitine as compared to using NaCl (42±7.5% versus 7.0±1.9%). In the case of using INRA-96, both for initial and final dilution, percentages of motile sperm were 59±12% at d 0 and 35±11% at d 7.

The replacement of NaCl by ChCl, L-histidine, and L-carnitine showed a change in the movement parameters. The curvilinear velocity (VCL) and tail beat cross frequency (BCF), as well as the amplitude of the lateral head movement (ALH), were higher when NaCl was replaced by ChCl. These values are exceeded by the replacement of ChCl by L-histidine and the combination of L-histidine and L-carnitine (Data not shown; see appendix).

4.2 Evaluation of the sperm intracellular sodium content

It was investigated if the intracellular sodium concentration was affected during storage in m-eBWWM with different NaCl, ChCl, L-histidine and L-carnitine contents. Therefore, after storage, sperm were stained with PI as well as SG. By looking at the presence or absence of PI (red) fluorescence, it was possible to discriminate between membrane intact and damaged sperm, whereas SG (green) fluorescence intensities were inspected as a measure for intracellular sodium contents.
In figure 4.3A, it can be seen that SG fluorescence intensities are numeric higher for membrane intact viable sperm when compared with membrane damaged dead sperm. Moreover, SG fluorescence histograms of membrane intact sperm indicated differences in the case of using different media for storage (figure 4.3B).

Mean and median SG fluorescence intensities of membrane intact sperm were derived from such histograms and exhibited similar trends. In figure 4.3C, it can be seen that at d 0 (directly after processing) sperm diluted in INRA-96 exhibited mean fluorescence intensities that were numeric 2.6±0.6-fold higher as found for sperm diluted in m-eBWWM, while no drastic changes were observed during storage (2.56±1.2 at d 1, 2.75±0.7 at d 3 and 2.6±0.5 at d 7). Also, no obvious changes were seen during storage in m-eBWWM. Interestingly, it was found that SG fluorescence intensities increased with substituting NaCl by ChCl. This can be seen at d 3 (NaCl: 1.1±0.7, NaCl/ChCl: 1.2±0.6, ChCl: 1.2±0.6), as well as d 7 (NaCl: 0.9±0.5; NaCl/ChCl: 1.2±0.4; ChCl: 1.8±1.1). In case the sperm were stored in m-eBWWM supplemented with L-histidine alone or in combination with L-carnitine, SG fluorescence intensities were numeric lowest (0.7±0.2 and 1.1±0.1 at d 7, respectively). It should be noted that SG fluorescence intensities exhibited great variation, especially in the case of high fluorescence intensities.

4.3 Effects of adding skim milk powder to m-eBWW media

It was tested if adding skim milk powder (SMP) to m-eBWWM affected sperm characteristics during long term storage. Therefore, following up the results described above, m-eBWW medium containing 100/100 mM L-histidine/L-carnitine (further
Figure 4.3 Flow cytometric analysis of intracellular sodium contents, as evaluated by sodium green fluorescence intensities of membrane intact and damaged/propidium iodide-stained sperm. Representative SG/green fluorescence intensity histogram overlays are shown in panels A and B, whereas a line plot on the relative units (r.u.) of mean SG fluorescence intensity versus storage duration is shown in panel C. Sperm were stored at 5°C for up to 7 d in m-eBWW media with different compositions. Panel A shows SG/green fluorescence intensity histograms of PI-stained/dead sperm (red histograms) versus PI-unstained/viable sperm (green histograms). Panel B shows histograms of (viable) sperm stored in m-eBWWM containing L-histidine and L-carnitine (blue histogram), NaCl (green histogram) or ChCl (red histogram). The m-eBWW media tested for determining mean viable sperm SG-fluorescence intensities versus storage time (panel C) contained NaCl/ChCl (mM) 95/0 (red line), 0/95 (blue line) or His/Car (mM) 100/100 (green symbols) mM. In addition, sperm was centrifuged, diluted and stored in INRA-96 (orange symbols). Mean values ± standard deviations are presented, which were calculated from six ejaculates from six different stallions.
referred to as m-eBWWM-6) was selected and supplemented with 0–5% SMP (figure 4.4).

The same medium was used for initial dilution, centrifugation, and storage. On the day of processing it can be seen that sperm membrane intactness and motility are negatively affected if using m-eBWWM-6 supplemented with 5% SMP, which likely is explained by the associated increase in the medium osmolality (from ~295 to ~490 mOsm). When looking at membrane intactness and motility, it seems like there is a beneficial effect of supplementing storage medium with increasing SMP concentrations reaching a maximum with adding 0.5–1.56% SMP. At d 7, the percentage of membrane intact sperm was 48±7.1% in case of 0% SMP compared to 67±3.1%, with 1.56% SMP, while motility percentages were respectively 26±9.8% and 30±15%. Motility percentages appeared slightly higher with using 0.5% SMP (31±12%; p>0.05).

4.4 Analysis of effects of medium osmolality on sperm characteristics

It appeared that the medium osmolality had a drastic effect on sperm viability; therefore, this was investigated in more detail. More specifically, it was tested if initial dilution with INRA-96 was beneficial (i.e. in terms of increasing osmotic tolerance limits) when compared with initial dilution in m-eBWWM-6 without further supplements or supplemented with 0.5% SMP. In both cases, the initial dilution and centrifugation were done in isotonic medium with an osmolality of ~300 mOsmol kg⁻¹.
Figure 4.4 Percentages of membrane intact (A,B) and motile (C,D: dark colors: total motility; light colors: progressively motile) sperm during storage for up to 7 d at 5°C in m-eBWWM-5 supplemented with skim milk powder ('Experiment 3'; see methods section for detailed descriptions). m-eBWWM media contained 100/100 mM His/Car without further supplements (green/yellow symbols) or skim milk powder was added at final concentrations of (%) 0.25 (white-light green symbols), 0.5 (light green symbols), 1.56 (green symbols), 2.5 (olive green symbols) and 5.0 (dark green symbols). For comparison, sperm was also stored in INRA-96 (orange symbols). Data are presented as line plots (A,C) as well as bar plots. Values marked with letters differ significantly (p<0.05) between diluents, whereas numbers differ significantly (p<0.05) between time point within a single diluent. Mean values ± standard deviations are presented, calculated from six ejaculates from six different stallions.
After centrifugation, the sperm pellet was resuspended in hypo- and hypertonic media (i.e. m-eBWWM diluted with water or supplemented with NaCl). Sperm motility was evaluated after 5 min of exposure (figure 4.5).

Sperm motility was numeric highest in isotonic m-eBWWM (59±8.3% and 67±6.7% with initial dilution in INRA-96 or m-eBWWM, respectively), whereas motility decreased upon exposure to hypo- and hyperosmolar conditions. Sperm motility, however, seemed relatively unaffected when exposed to medium osmolality was ranging from 250–350 mOsmol kg⁻¹. In all cases, sperm motility percentage was 6–10% higher if INRA-96 was used as the first diluent. In the case of exposure to 205 mOsmol kg⁻¹, 65±6.5% motile sperm were found when the initial dilution was with INRA-96. Semen first diluted in m-eBWWM showed 45±13 % motile sperm.

It should be noted that only membrane intact sperm are motile, and can respond osmotically. In figure 4.5A, micrographs are shown of eosin-stained sperm after exposure to 110 mOsm kg⁻¹ conditions. This nicely illustrates that membrane intact sperm not exhibiting eosin-uptake/staining exhibit tail rolling and loops due to water uptake.

4.5 Possible beneficial effects of supplementing m-eBWWM with the antioxidant coenzyme Q10 or with polyvinyl alcohol

We hypothesized that supplementing m-eBWWM with the antioxidant coenzyme Q10 could have an impact on sperm motility and membrane intactness (i.e. increase survival rates during cooled storage). Therefore, sperm survival was monitored after dilution and storage in m-eBWWM-6 containing 0.5% SMP as well as 0.75 mM coenzyme Q10. In a further experiment, the possible beneficial effects of
adding 0.1% polyvinyl alcohol (PVA) were tested. Although both experiments were performed separately each with their own controls (i.e. use of INRA-96 for primary and final dilution), for easy comparison data are presented graphically in the same figure (figure 4.6). It was found that both the addition of coenzyme Q10 or PVA had no significant (p>0.05) effects on sperm viability on the day of processing. Only minor effects on sperm motility were seen during cooled storage.

After 3/7 d storage at 5°C, slightly higher percentages of motile sperm were found in case of adding Q10 (d 3: 36±5.4% versus 43±1.1%, d 7: 30±3.3% versus 35±12%; p>0.05) but not with PVA (d 7: 27±15% vs 28 ±14%; p>0.05). In both cases, membrane intactness was not affected. As described above, sperm viability was higher if using INRA-96 for initial dilution and storage.

4.6 Effects of supplementing sperm processing and preservation media with antibiotics

It was investigated if antibiotics (AB) in the medium used for sperm storage could be omitted. Therefore, sperm was stored in medium without AB, after dilution in INRA-96 or m-eBWWM-6 with(out) AB. Commercially available INRA-96 contains penicillin, gentamicin, and amphotericin B, whereas in-house prepared m-eBWWM-6 was prepared without AB as well as supplemented with antibiotics. In the latter case, the antibiotics penicillin, gentamicin, streptomycin and nystatin were added. Details on used antibiotics and concentrations are given in table 3.2 and table 3.3. Sperm membrane intactness and motility were investigated during 7 d storage at temperatures ranging from 5−30°C (figure 4.7 and 4.8).
Figure 4.5 Sperm were exposed to medium of different osmolalities, after which they were observed microscopically (A) and motility characteristics were evaluated (B). Panel A shows micrographs of sperm exposed to 100 mOsmol kg\(^{-1}\) m-eBWWM-6 solution, after which they were stained with nigrosine-eosin and a dried smear was prepared and observed. Membrane damaged sperm exhibit eosin-stained sperm heads (marked with an asterisk), whereas membrane intact sperm exhibit various forms of tail rolling/loops formed due to their osmotic response. In panel B, percentages of motile sperm are plotted as a function of the medium osmolality they were exposed to for 10 min at 38°C (see methods section 3.4 for detailed descriptions). Hypotonic media were prepared by diluting m-eBWWM-6 with water, whereas hypertonic media were prepared by adding NaCl. Sperm were exposed to media of different osmolalities after 2 h storage at 5°C in either INRA-96 (blue line) or m-eBWWM6 with 0.5% skim milk powder (green line). Mean values ± standard deviations are presented, calculated from six ejaculates from six different stallions.
Figure 4.6 Percentages of membrane intact (A,B) and motile (C,D: dark colors: total motility; light colors: progressively motile) sperm during storage for up to 7 d at 5°C in m-eBWWM-6 containing 100/100 mM His/Car without (blue bars), with supplementation of 75 μM coenzyme Q-10 (bars with olive green diagonals) or 0.1% (w/v) PVA (bar with yellow diagonals). INRA-96 (orange bars) was used for dilution of raw semen, and sperm was resuspended in m-eBWWM after centrifugation (see methods section for detailed descriptions). Data are presented as line plots (A,C) as well as bar plots (B,D). Values marked with letters differ significantly (p<0.05) between diluents, whereas numbers differ significantly (p<0.05) between time point within a single diluent. Mean values ± standard deviations are presented, calculated from six ejaculates from six different stallions.
At the day of semen collection and processing, percentages of membrane intact and motile sperm were not affected by using either m-eBWWM-6 with or without AB as a first diluent before dilution in the medium for storage.

During storage for up to 7 d percentages of membrane intact and motile sperm decreased, which occurred to a greater extent when stored at higher temperatures (i.e. above room temperature; p<0.05). The temperature-dependent decrease in sperm membrane intactness was less severe in the case of using INRA-96 as a first diluent. In the case of using INRA-96 as the primary diluent, after 7 d storage at 5 or 17°C higher (p<0.05) percentages of membrane intact sperm were found. Percentages were higher irrespective of the absence or presence of AB in the m-eBWWM-6 storage medium. The use of m-eBWWM as the first diluent resulted in significantly lower membrane intact sperm after 7 d (p<0.05). When using m-eBWWM-6 with AB, both as a first and second diluent, percentages of membrane intact sperm decreased significantly (p<0.05) from 79±3.2% at the day of semen collection to 53±12% and 51±15% after 7d at 5°C and 17°C, respectively. If stored for 7 d at 5°C, percentages of membrane intact sperm were slightly higher in the case of using m-BWWM-6 without AB (58±6.8%; p>0.05). However, if stored at 17°C, the decrease in sperm membrane intactness was higher if the m-eBWWM formulation contained no antibiotics (33±17%; p<0.05).

If looking at sperm motility values during storage in AB-free m-eBWWM-6, using INRA-96 as the first diluent resulted in significantly (p<0.05) higher values as when using m-eBWWM-6 with AB (figure 4.7 versus 4.8). Moreover, with exposure to increasing temperature, negative temperature effects during storage in m-eBWWM appeared less pronounced with using INRA-96 as the first diluent.
In figure 4.9 is illustrated that using m-eBWWM-6 with AB prior to storage in m-eBWWM-6 without AB also results in higher sperm viability as compared to using m-eBWWM-6 without AB for initial dilution as well as storage. The effect is especially true for storage at higher temperatures. After 7 d storage at 17°C, initial dilution without AB resulted in 5.7±1.0% motile sperm, which was significantly (p<0.05) increased to 23±7.2% in the case of using AB in the initial diluent. The same trend was seen for percentages of membrane intact sperm (32±15% versus 55±7.9%; p<0.05).

For storage in the AB-free medium at 5°C, sperm viability values were similar for using m-eBWWM-6 with or without AB for initial dilution. In addition to progressive and total motility, local circular motility and movement parameters such as VCL, BCF, and ALH were also measured in the experiments.

On average, at day of semen collection considerably higher numbers of local circular motile sperm were found in m-eBWWM-6 with or without antibiotics compared to INRA-96 when using the same medium for first and second dilution (1.7±1.7% versus 8.0±2.1% and 8.2±1.1%, respectively). From day 3 of storage, no difference could be observed but the values decrease significantly (p<0.05) over the course of the storage period. The comparison of INRA-96 with m-eBWWM-6 with and without antibiotic supplementation showed higher VSL values on the day of semen collection and processing for samples diluted with m-eBWWM-6. From the third day of storage, the differences between the diluents used are small (Data not shown; see appendix). The parameters of straightness (STR) and linearity (LIN) show only a small difference between the used diluents over the whole storage period of 7 days.
Figure 4.7 Percentages of membrane intact (B) and motile (D; dark colors: total motility; light colors: progressively motile) sperm during storage for up 7 d at different storage temperatures ranging from 5–30°C. The same diluent was used for initial dilution and resuspension after centrifugation and storage. The media that were tested included INRA-96 (orange bars), and m-eBWW-6 with AB added (bar with light blue diagonals) or no AB added (dark blue bars) (‘Experiment 4’; see methods section for detailed descriptions). For illustrating the effect of the storage temperature more clearly, for 3 d storage, data are also presented as line plots as a function of the temperature (A: sperm membrane intactness, C: total motility). Values marked with letters differ significantly (p<0.05) between diluents, whereas numbers differ significantly (p<0.05) between time point within a single diluent. Mean values ± standard deviations are presented, calculated from six ejaculates from six different stallions.
Figure 4.8 Percentages of membrane intact (A) and motile (B; dark colors: total motility; light colors: progressively motile) sperm during storage for up to 7 d at different storage temperatures ranging from 5−30°C. Directly after collection, sperm were diluted in INRA-96, after which samples were centrifuged and resuspended in INRA-96 (orange bars), m-eBWW-6 with AB added (bars with light blue diagonals) or no AB added (dark blue bars) (‘Experiment 5’; see methods section for detailed descriptions). For illustrating the effect of the storage temperature more clearly, for 3 d storage, data are also presented as line plots as a function of the temperature (C: sperm membrane intactness, D: total motility). Values marked with letters differ significantly (p<0.05) between diluents, whereas numbers differ significantly (p<0.05) between time point within a single diluent. Mean values ± standard deviations are presented, calculated from six ejaculates from six different stallions.
Figure 4.9 Percentages of membrane intact (A–C) and motile (D; dark colors: total motility; light colors: progressively motile) sperm during storage for up 7 d at 5°C and 17°C. After collection, semen was diluted with m-eBWWM-6 with (cyan blue stripped) or without (dark blue) AB after which sperm was stored in AB-free m-eBWWM-6 (‘Experiment 7’; see methods section for detailed descriptions). Data are presented as line (A,C; membrane intactness versus storage duration at 5 and 17°C, respectively) and bar plots (B,D). Values marked with letters differ significantly (p<0.05) between diluents, whereas numbers differ significantly (p<0.05) between time point within a single diluent. Mean values ± standard deviations are presented, calculated from six ejaculates from six different stallions.
4.7 Evaluation of microbial growth in sperm samples stored at different temperatures with and without the addition of antibiotics

For part of the incubations described above, microbial growth was evaluated during storage. Particular emphasis was on detecting microbial growth during storage in an AB-free medium. In figure 4.10, it can be seen that numbers of microbial counts detected at d 0 are in the similar range or lower as detected in raw semen. These samples, however, are difficult to compare directly. Centrifugation, removal of seminal plasma and dilution to a lower sperm concentration affect the obtained results (but likely decreases the number of microbes). Moreover, it can be seen that values only slightly increased during 7 d storage at 5°C, whereas this increase was higher in the case of storage at 17°C. Compared to values determined at d 0 (i.e. directly after processing) the relative numeric increase in CFU per square centimeter was 1.7–5-fold and even more than 10-fold, respectively. If sperm was diluted in medium containing AB before storage in an AB-free medium, this appeared to be sufficient for diminishing the microbial count in sperm samples stored for 7 d at 5°C as well as 17°C. Dilution and centrifugation of fresh stallion semen resulted in the bisection of CFU per square centimeter compared to native semen (mean value 74.9 and 35.7, respectively). During the cultivation of the sperm samples, the growth of only one scattered mold was perceived.
4.8 DNA defragmentation index (DFI) as a parameter to detect fertility of stored semen

For the samples that are presented in figure 4.10, also DFI-values were determined (figure 4.11). The determination of chromatin integrity was done to obtain insights in the effects of storage conditions on sperm chromatin structure and fertilizing capacity. On the day of semen collection, DFI-values were determined to be 7.43±3.1% and 11±6.0%, respectively, for sperm finally diluted in m-eBWWM with and without AB. With increasing storage duration, DFI-values increased (p<0.05). After 3 d storage, this increase appeared larger for specimens stored at 17°C as compared to storage at 5°C, whereas after 7 d storage differences seemed less clear. DFI-values for samples diluted with INRA-96 seemed numeric lower. Higher DFI-values were also seen for samples diluted in m-eBWWM-6 with AB, but values appeared lower as those determined for specimens diluted in m-eBWWM-6 without antibiotics (p>0.05). It should be noted, however, that diluents may interfere and affect the SCSA outcome. After 7 d storage at 5°C, DFI-values ranged from 19–23%, irrespective of the diluent used. DFI-values after 7 d storage at 17°C were 19±6.8% if stored in INRA-96, and 27±15% and 20±4.7% if stored in m-eBWWM-6 respectively without and with AB.
Figure 4.10 Analysis of microbial counts in raw semen (A,B) as well as sperm samples that were stored for up to 7 d after different processing approaches (C–F; i.e. using AB containing/free diluents). See details in the description of ‘Experiment 7’. Representative photographs of ‘Easicult’ test slides are presented (A,C,D) as obtained using conditions given by the manufacturer. Furthermore is presented a graphical presentation on derived factor values on colony forming units per mL (B,E,F). Media that were used for initial dilution (INRA-96, m-eBWWM-6 with/out AB), as well as those used for resuspension and storage after centrifugation (i.e. m-eBWWM-6 without AB for all cases), are indicated below the respective panels. In panels E and F, values are presented for diluting and storing sperm in m-eBWWM-6 without AB, if stored at respectively 5 and 17°C. Data for all stallions are indicated with separate circles (i.e. n=6 for raw semen, n=3 for stored sperm samples).
Figure 4.11 DFI-values, as determined by SCSA, for stallion sperm stored in m-eBWWM-6 without AB after initial dilution in INRA-96 (blue bars with orange diagonals), m-eBWWM-6 without (dark blue bars) and with AB (bars with light blue diagonal); for up to 7 d at 5°C (A) or 17°C (B). See details in the description of 'Experiment 7'. Values marked with letters differ significantly (p<0.05) between diluents, whereas numbers differ significantly (p<0.05) between time point within a single diluent. Mean values ± standard deviations are presented, calculated from six ejaculates from six different stallions.
5 DISCUSSION AND CONCLUSIONS

5.1 Replacing NaCl by ChCl or L-histidine and/or L-carnitine results in higher sperm viability during storage

In the first part of this study, it was found that stallion sperm viability during cooled storage was increased by using a modified extender, in which NaCl was replaced by ChCl, L-histidine, and/or L-carnitine. The medium osmolality was kept the same. Percentages were highest using a combination of L-histidine and L-carnitine. This has been earlier described by GIBB et al. (2016). Replacing NaCl by ChCl likely has a beneficial effect due to the reduced action of the Na-dependent ATPases. With higher sodium levels and ATP/energy consumption, sperm viability decreases faster during storage. It has been reported before, that a high enough level of mitochondrial ATP is required for maintaining stallion sperm membrane intactness and viability (PLAZA-DAVILA et al. 2016). Whereas NaCl can rapidly move across the membrane, this is slower in case of ChCl or L-histidine and L-carnitine.

The increased sperm viability in case of replacing NaCl by histidine/carnitine is explained by the fact that these compounds can also serve as energy sources (GIBB et al. 2015). While maintaining the osmotic balance, the combination of L-histidine and L-carnitine was more beneficial as compared to using L-histidine alone. High concentrations of L-histidine possibly have adverse effects, causing ‘toxicity’, or there is only a limited uptake and metabolism of L-histidine. In addition, carnitine has an important role in the beta-oxidation of fatty acids, whereas carnitine is needed for
transfer of the acyl-part into the mitochondria to generate acetyl-CoA (STEIBER et al. 2004).

Since histidine/carnitine support the energy metabolism, this may affect sperm motility characteristics. When inspecting sperm movement characteristics (such as VCL, BCF, and ALH) it was actually found that in the presence of histidine/carnitine sperm exhibited higher VCL values and looked ‘hyperactive’. Hyperactive motility is normally characterized by both increased VCL and ALH values (RATHI et al. 2001).

5.2 Supplementation of skim milk powder improved sperm viability, whereas coenzyme Q10 or PVA had no effect on evaluated sperm characteristics

Diluting semen directly after collection in a milk-containing medium, like INRA-96, appeared beneficial above using m-eBWWM without milk components for initial dilution; as was evident from sperm membrane integrity and motility values. Also, it was found that stallion sperm viability increased when 0.5−1.56% (w/v) SMP was added to m-eBWWM and used for initial dilution. The beneficial effect of adding SMP to stallion semen extenders is well known (KENNEY et al. 1975). Milk compounds have been described to protect sperm via anti-oxidative function and preventing membrane lipid structural changes (KATILA 1997; MANJUNATH et al. 2002). SMP contents below 0.5% (w/v) are not sufficient to protect the sperm membrane. Adding too high contents of SMP, on the other hand, coincides with increasing the medium osmolality due to the salts also present in SMP.

Furthermore, we tested if supplementing m-eBWWM with the antioxidant coenzyme Q10 or addition of PVA affected sperm viability. In our hands, adding
coenzyme Q10 or PVA had no effects on sperm viability during 7 d storage at 5°C. There are, however, several reports in which beneficial effects are described, and adding such compounds to semen extenders increased sperm viability during storage. For example, a positive effect on sperm motility in men has been described (LAFUENTE et al. 2013; GIACONE et al. 2017). In stallions, improved sperm motility and fertility in case of adding coenzyme Q10 has been reported in response to cold-shock (CARNEIRO et al. 2018). It has also been reported, that addition of PVA preserves sperm motility and viability (BAVISTER 1981), especially when fatty acids (FA) are present in the extender (HOSSAIN et al. 2007). In this study, addition of PVA did not have a significant effect on sperm viability, which can be explained by a lack of FA in the ejaculate and the used diluent.

5.3 Diluent m-eBWWM-6 is suitable for sperm storage for 7 d at 17°C

During storage for up to 7 d, percentages of membrane intact and motile sperm decreased, which occurred to a greater extent when stored at higher temperatures (i.e. above room temperature). We found that the temperature-dependent decrease in sperm membrane intactness, as well as sperm motility, was less severe when using INRA-96 as the first diluent as compared to using m-eBWWM not containing milk components. Moreover, after initial dilution in INRA-96, storage at 17°C seemed feasible (VIDAMENT et al. 2012). The storage duration at 17°C could be prolonged for up to 7 d when stored in m-eBWWM-6 after initial dilution in INRA-96. When the initial dilution was also done in m-eBWWM-6, only 3 d storage at 17°C seemed feasible, whereas storage durations up to 7 d were possible
if stored at 5°C. The detection of deteriorated semen quality and viability within 3 d storage at higher temperatures has been reported before (SQUIRES et al. 1988; FREITAS-DELL’AQUA et al. 2012).

5.4 Semen can be stored at 5°C for 7 d in m-eBWWM-6 without antibiotics

In case m-eBWWM-6 without antibiotics was used as initial and storage diluent, and samples were stored for up to 7 d, sperm viability decreased and numbers of CFU per square centimeter increased. These effects were more pronounced if storage was done at higher temperatures (i.e. 17 versus 5°C). Depending on species, and toxic components produced by them, bacteria can affect sperm viability. Lipopolysaccharides as well as porins may interact with the sperm membranes affecting viability (GORGA et al. 1992; AURICH and SPERGSER 2007). It should be noted that, as done in the current study, the bacterial load of fresh stallion semen is drastically reduced in case semen samples are subjected to centrifugation (MORRELL et al. 2014; GUIMARAES et al. 2015; VARELA et al. 2018).

Interestingly, percentages of membrane intact sperm were found to be higher in the case of using m-BWWM-6 without AB for storage at 5°C for 7 d. A similar observation has been described by AURICH and SPENGSER (2007). Especially the presence of high AB concentrations negatively affect sperm viability (JASKO et al. 1993).
5.5 Presence or absence of antibiotics in the storage medium did not impair sperm fertility

In the current study, DFI-values were analyzed as a measure for male fertility. Male fertility strongly correlates with DFI-values (LOVE and KENNEY 1998). In case AB were omitted, higher DFI values were found, especially for storage at higher storage temperatures. It should be noted, however, that diluents like INRA-96, may interfere and affect the SCSA outcome.

Fertile stallions are described to have an average DFI value of 12%, while subfertile and infertile stallions had DFI values of approximately 17% and 25%, respectively (LOVE 2005). Based on this classification, and the DFI-values determined in this study, it can be assumed that fertility is not drastically affected by the processing nor storage conditions tested in this study. Determined DFI-values of sperm samples increased when stored at higher storage temperatures (i.e. 17°C and above) or for longer storage durations (i.e. longer than 3 d), but were still below ~32%.

5.6 Conclusions

In this study it was shown that (1) sperm viability during cooled storage could be increased by using a medium in which NaCl was replaced with ChCl or L-histidine and/or L-carnitine. Furthermore, (2) sperm viability and storage stability was increased by adding ~0.5% SMP, whereas adding coenzyme Q10 or PVA did not appear to have an effect on sperm viability during storage. If using m-eBWWM-6 as a
diluent, (3) storage for 7 d at 5°C seemed feasible for maintaining sperm viability, as well as 3 d storage at 17°C. Finally, it was found that (4) presence of antibiotics in the first dilution is sufficient to prevent bacterial growth during storage in antibiotics-free diluent for 7 days at 5°C. Fertility of semen stored with(out) antibiotics, was not affected since DFI values showed no drastically differences amongst the semen processing nor storage conditions tested.
6 SUMMARY

Yana Müller (2019)

Effects of substituting sodium chloride and omitting antibiotics in stallion semen diluents for prolonged storage at elevated temperatures

In the horse breeding industry, most of the warmblood mares are inseminated via artificial insemination. Raw semen can be used only for a limited period of time for artificial insemination. Sperm longevity, however, can be prolonged when diluting in specific ‘extenders’. Typical extenders are composed of physiological salts, nutrients, buffering compounds, protective agents, and agents that counteract microbial growth (i.e. antibiotics and antifungals). The aims of the studies described in this thesis were to: (1) investigate sperm viability during storage in a medium of a defined osmolality with varying NaCl, ChCl, L-histidine and L-carnitine concentrations, (2) determine effects of adding skim milk powder, coenzyme Q10 and polyvinyl alcohol to the storage medium, (3) investigate stallion sperm viability during storage for up to 7 d at temperatures ranging from 5–30°C, and (4) determine if diluents without antibiotics can be used for storing stallion semen at 5 and 17°C.

In the first part of this study, sperm viability was investigated during storage using diluents that were modifications of Biggers, Whitten and Whittingham Medium, originally described elsewhere. Modified equine BWW media (m-eBWWM) were prepared with varying NaCl and ChCl concentrations (95/0, 47.5/47.5, 0/95 mM) as well as NaCl replacement by L-histidine and/or L-carnitine (200/0, 100/100 mM) concentrations. Increased intracellular sodium concentrations typically result in the
activation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPases to maintenance homeostasis under ATP consumption. It could be shown that sperm viability during cooled storage could be increased by using a medium in which NaCl was replaced with ChCl or L-histidine and/or L-carnitine. Highest percentages of membrane intact and motile sperm were found using L-histidine and L-carnitine.

After finding that m-eBWW medium in which NaCl was replaced by L-histidine and L-carnitine preserves sperm viability only after initial dilution in milk-containing extender, the effect of adding skim milk powder (SMP) was investigated. In addition, possible beneficial effects of also adding coenzyme Q10 and PVA were evaluated. We found that adding 0.5% SMP to m-eBWWM increased sperm viability whereas supplementation with coenzyme Q10 and PVA showed no significant effects on sperm viability during 7 d storage at 5°C or 17°C.

Sperm stored at 5°C are exposed to cold-shock, which is evident as decreased motility and membrane intactness. In order to prevent cold shock, it was investigated if sperm could be stored at higher temperatures when using m-eBWW. Sperm viability was therefore followed during storage for up to 7 d at temperatures ranging from 5–30°C. If using m-eBWWM containing histidine/carnitine and SMP, as a diluent, sperm preservation and storage for 7 d at 5°C seemed feasible, as well as 3 d storage at 17°C. Anyways, during storage percentages of membrane intact and motile sperm decreased, which occurred to a greater extent when stored at higher temperatures.

In the last part of this study, the impact of antibiotics supplementation on sperm viability during long-term storage at various temperatures was evaluated. It could be shown that the presence of antibiotics in the first diluent is sufficient to
prevent bacterial growth during storage in antibiotics-free diluent for 7 d at 5°C. Furthermore, as a measure for chromatin intactness and fertility, the susceptibility of sperm DNA to acid denaturation was analyzed. Derived DNA fragmentation index values showed no drastic differences amongst the semen processing nor storage conditions tested.

Taken together, in the current study it is shown that a m-eBWW medium in which NaCl is substituted with L-histidine and L-carnitine, supplemented with 0.5% SMP preserves sperm viability during 7 d storage at 5°C or 3 d storage at 17°C. Furthermore, presence of antibiotics in the diluent used for initial dilution and centrifugation appeared sufficient for preventing bacterial growth during storage in antibiotics-free diluent.
7 ZUSAMMENFASSUNG

Yana Müller (2019)

Auswirkungen des Ersatzes von Natriumchlorid und des Verzichts von Antibiotika in Medien für die Langzeitlagerung von Hengstsperma bei erhöhten Temperaturen

Verdünner zur Lagerung von Hengstsamen ohne den Zusatz von Antibiotika bei 5°C und 17°C verwendet werden kann.

Im ersten Teil dieser Studie wurde die Spermienvitalität während der Lagerung mit Verdünnern, die Modifikationen des m-eBWWM waren, untersucht. Diese m-eBWW Medien wurden mit unterschiedlichen Konzentrationen von NaCl, ChCl (95/0, 47,5/47,5, 0/95 mM) oder L-Histidin und/oder L-Carnitin (200/0, 100/100 mM) hergestellt. Erhöhte intrazelluläre Natriumkonzentrationen führen typischerweise zur Aktivierung von Na⁺-K⁺-ATPasen, um die Homöostase aufrechtzuerhalten; dies geschieht unter Verbrauch von ATP. Es konnte gezeigt werden, dass die Spermienvitalität während der gekühlten Lagerung durch die Verwendung eines Mediums erhöht werden kann, in welchem NaCl entweder durch ChCl oder L-Histidin und/oder L-Carnitin ersetzt wurde. Die höchsten Prozentsätze motiler Spermien und Spermien mit intakter Plasmamembran wurden bei der Verwendung von L-Histidin in der Kombination mit L-Carnitin gemessen.

Nachdem festgestellt wurde, dass die Spermienvitalität nach einer ersten Verdünnung in einem magermilchhaltigen Verdünner im m-eBWW-Medium mit L-Histidin/L-Carnitin besser aufrecht erhalten werden konnte, wurde der Effekt der Zugabe von Magermilchpulver (SMP) untersucht. Darüber hinaus wurden mögliche positive Effekte der Zugabe von Coenzym Q10 und PVA untersucht. Die Zugabe von 0,5% SMP zum m-eBWW-Medium erhöhte die Spermienvitalität, während die Supplementierung mit Coenzym Q10 und PVA keine signifikanten Auswirkungen auf die Vitalität der Spermien während der Lagerung für bis zu 7 Tage bei 5°C oder 17°C zeigte.
Spermien, die bei 5°C gelagert werden, sind einem Kälteschock ausgesetzt, welcher sich in vermindelter Beweglichkeit und Membranintegrität zeigt. Um einen Kälteschock zu vermeiden, wurde untersucht, ob Spermien nach der Verdünnung mit m-eBWWM bei höheren Temperaturen gelagert werden können. Die Spermienvitalität wurde daher während der Lagerung für bis zu 7 Tage bei Temperaturen von 5–30°C verfolgt. Bei der Verwendung von m-eBWWM mit L-Histidin, L-Carnitin und SMP schien eine Konservierung und Lagerung der Spermien für 7 Tage bei 5°C sowie eine dreitägige Lagerung bei 17°C möglich. So nahmen die Prozentsätze membranintakter und beweglicher Spermien während der siebentägigen Lagerung zwar ab, was während der Lagerung bei höheren Temperaturen jedoch stärker der Fall war.

Im letzten Teil dieser Arbeit wurde der Einfluss der Antibiotika-supplementierung auf die Spermienvitalität während der Langzeitlagerung bei verschiedenen Temperaturen untersucht. Es konnte gezeigt werden, dass das Vorhandensein von Antibiotika im ersten Zentrifugations-Verdünnungsmedium ausreichend ist, um das Bakterienwachstum während der Lagerung in einem antibiotikafreien Verdünner für 7 Tage bei 5°C zu verhindern. Darüber hinaus wurde die Anfälligkeit der Spermien-DNA für eine Denaturierung durch Säure analysiert, die als Maß für die Intaktheit von Chromatin und die Befruchtungsfähigkeit der gelagerten Spermien dient. Der so ermittelte DNA-Fragmentierungsindex zeigte keine drastischen Unterschiede zwischen der Samenverarbeitung und den getesteten Lagerungsbedingungen.

Zusammenfassend wird in der aktuellen Studie gezeigt, dass ein m-eBWW-Medium, in dem NaCl durch L-Histidin und L-Carnitin ersetzt wurde, ergänzt mit 0,5%
SMP, die Spermienvitalität bei der Lagerung für 7 Tage bei 5°C oder für 3 Tage bei 17°C bewahrt. Darüber hinaus schien das Vorhandensein von Antibiotika in dem für die Erstverdünnung und Zentrifugation verwendeten Verdünner auszureichen, um das Bakterienwachstum während der Lagerung in einem antibiotikafreien Verdünner zu verhindern.
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Figure 9.1 Percentages of VCL (μm; A), BCF (Hz; B) and ALH (μm; C) during storage of sperm for up to 7 d at 5°C in m-eBWWM-1–5 with varying NaCl, ChCl, L-histidine and L-carnitine contents ('Experiment 1'; see methods section for detailed descriptions). Directly after collection, sperm were diluted in INRA-96 or m-eBWWM-1–5, after which samples were centrifuged and resuspended in the fresh medium of the same composition for cooled storage. The m-eBWW media contained NaCl/ChCl (mM) 95/0 (red lines), 47.5/47.5 (lilac lines), 0/95 (blue lines) or His/Car (mM) 200/0 (green lines), 100/100 (yellow lines) mM. For comparison, sperm were also stored in INRA-96 (orange lines). Data are presented as line plots. Mean values ± standard deviations are presented, which were calculated from six ejaculates from six different stallions.
Figure 9.2 Percentages of VSL (μm sec⁻¹; A), STR (%; B) and LIN (%; C) during storage of sperm for up to 7 d at 5°C in m-eBWWM-6 with or without addition of antibiotics ('Experiment 5'; see methods section for detailed descriptions). Directly after collection, sperm were diluted in m-eBWWM-6 with (light blue lines) or without (dark blue lines) antibiotics, after which samples were centrifuged and resuspended in the fresh medium of the same composition for cooled storage. For comparison, sperm were also stored in INRA-96 (orange lines). Data are presented as line plots. Mean values ± standard deviations are presented, which were calculated from six ejaculates from six different stallions.
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**Figure 3.1** Schematic presentation of the experimental design of ‘Experiment 1’ and ‘Experiment 2’ determining effects of first diluent during cooled storage for up to 7d.

**Figure 3.2** Schematic presentation of the experimental design of ‘Experiment 3’, which was performed to determine effects of supplementation of SMP and PVA.

**Figure 3.3** Schematic presentation of the experimental design of ‘Experiment 5’ and ‘Experiment 6’ to verify possibility to store diluted sperm without antibacterial additive.

**Figure 4.1** Percentages of membrane intact and motile sperm during long-term cooled storage in m-eBWWM-1−5 with varying NaCl, ChCl, L-histidine and L-carnitine contents.

**Figure 4.2** Percentages of membrane intact and motile sperm during long-term cooled storage in m-eBWWM-1−5 with varying NaCl, ChCl, L-histidine and L-carnitine contents after first dilution in INRA-96.

**Figure 4.3** Flow cytometric analysis presenting values of intracellular sodium contents, as evaluated by sodium green fluorescence intensities of membrane intact and damaged/propidium iodide-stained sperm.

**Figure 4.4** Sperm characteristics after long-term cooled storage in m-eBWWM-5 supplemented with skim milk powder.

**Figure 4.5** Reaction of sperm which were exposed to medium of different osmolalities.

**Figure 4.6** Sperm characteristics after long-term cooled storage in m-eBWWM-5 supplemented with coenzyme Q10 or PVA.

**Figure 4.7** Percentages of membrane intact and motile sperm during long-term storage at different storage temperatures ranging from 5−30°C. The same diluent was used for initial dilution and resuspension after centrifugation and storage.

**Figure 4.8** Percentages of membrane intact and motile sperm during long-term storage at different storage temperatures ranging from 5−30°C after first dilution in INRA-96.

**Figure 4.9** Sperm characteristics of sperm during long-term storage at 5°C and 17°C to verify possibility to store diluted sperm without antibacterial additive.

**Figure 4.10** Analysis of microbial counts in raw as well as stored sperm samples after different processing approaches.

**Figure 4.11** DFI-values for stallion sperm stored in m-eBWWM-6 without AB after initial dilution in INRA-96, m-eBWWM-6 without and with AB.
9.4 List of tables

Table 3.1  List of stock solutions used for preparing different m-eBWW media
Table 3.2  Table summarizing the m-eBWWM formulations tested
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10 DANKSAGUNG

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