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# Energy Management of Mature Mammalian Spermatozoa

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Additional information is available at the end of the chapter

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## 1. Introduction

The ultimate goal of a mammalian sperm is the transmission of paternal genome to the next generation. To achieve this goal, mammalian spermatozoa are very specialized cells, which have a precise cellular design in dependence on the evolutionary reproductive strategy chosen by each species. This specificity is a very important point, since aspects such as the exact point of ejaculate deposition, oestrous time-lapse, number of males mated with a single female and number and sequence of oocytes released in a single ovulation will be key factors in the modulation of sperm function in order to yield optimal “in vivo” fertility rates. A practical consequence of this specificity is that the functional features that distinguish sperm from one species cannot be extrapolated to other species, hindering thus the assumption of an overall picture to explain mammalian sperm function. Furthermore, the extraordinary complexity of the molecular mechanisms implied in the control and modulation of mammalian mature sperm functions makes impossible to a complete description of these mechanisms in the limited space of this chapter. In this way, this chapter will be devoted to a succinct overview of the mechanisms by which mature mammalian sperm manage their energy levels, with a special emphasis in the observed differences among species and also during their entire life span of sperm from ejaculation. For this purpose, this chapter is centered in the description of specific, very important and punctual aspects of sperm energy metabolism. The first aspect is the type of energy sources, both external and internal, that mammalian sperm can utilize to obtain energy. The second aspect is centered in the main metabolic pathways that mammalian sperm utilize to obtain energy, as well as in the basic control mechanisms that modulate these pathways. The third point will involve the precise role that mitochondria play in the control of the overall mammalian sperm function. Finally, the fourth and last point will be focused in the existence of separate metabolic mammalian

sperm phenotypes as the result of the precise evolutionary strategy launched by each species to optimize fertility.

## **2. Mature mammalian spermatozoon: a dynamic cell with changing energy necessities during its lifetime.**

A common characteristic of mature mammalian sperm among species is that these cells are dynamic structures, which must underlie dramatic functional changes during their entire life span, from ejaculation to syngamia. These functional changes, in turn, will imply equally dramatic changes in all aspects of sperm energy management, from external energy sources to energy-consuming functions such as specific motion patterns or capacitation-linked cellular and membrane changes. Thus, a succinct description of the most important changes of mammalian sperm function from ejaculation is needed to a better understanding of the observed changes in sperm energy management during their life span.

Ejaculation implies the launching of a rapid succession of events that completely changes sperm physiology. Thus, ejaculated spermatozoa acquire a fast motion pattern; which is accompanied with several changes in cell membrane composition. The main responsible for these changes are seminal plasma, which contacts with spermatozoa during ejaculation. Composition of seminal plasma is complex. Even worse, seminal plasma has a totally different composition when comparing among different species. An example of this is the monosaccharide composition of seminal plasma. We can detect a wide variety of different sugars, such as glucose, fructose and sorbitol. Moreover, the concentration of these sugars is completely different. Thus, whereas fructose is the main sugar in species like human, glucose is present in significant amount in species like boar [48]. The main monosaccharide present in horse seminal plasma is, at the contrary, sorbitol [42], whereas species like dog has not any monosaccharide in significant concentrations [44, 48]. A similar pattern can be found when analyzing seminal plasma proteins. Thus, whereas dog has practically only one protein, which is characterized by its arginine esterase activity [10], other species such as boar and ram has a wide variety of proteins, including a membrane-protective protein family [8, 56].

What could be the main reason for the enormous differences in composition among seminal plasma from separate species? Investigators can only speculate regarding this point. However, it would be reasonable to suppose that the main reason for these differences is the very specific evolutionary reproductive strategies developed in each species to optimize their fertilizing abilities. In this way, it is logical to suppose that seminal plasma of one long-lived species like dog, would have completely separate characteristics to the seminal plasma of other shorter-lived species, like bull. It is noteworthy that dog spermatozoa have to survive for relatively long periods inside the bitch genital tract and, moreover, must be prepared to compete against spermatozoa from other individuals. On the contrary, bull spermatozoa is adapted to a shorter life-span, since the time lapse between ejaculation and cow ovulation is very short indeed. In any case, seminal plasma has some common features among species intended to solve common problems that spermatozoa find after ejaculation. Thus, seminal

plasma must contain components that activate sperm motility. This is absolutely essential in species in which ejaculation is carried out either at the vaginal vestibulum or at cervix. In these placements, the female genital tract presents a very active immunological system, which is further activated during oestrus [23]. This very active system will eliminate all spermatozoa that would not be enough fast or enough fortunate to leave the area and, in this way, sperm motility must be activated immediately after ejaculation. A wide array of seminal plasma components have been identified as motility activators. From these, probably the most known are prostaglandins, which have been found as a common seminal plasma component in several species like human and bovine [25, 52], although there are other components that plays a role as motility activators. Regarding prostaglandins, it has been described that their motility activation role is not mediated by receptors [49]. The activation of this non-receptor pathway would evolve the activation of specific energy-consuming pathways, pointing thus the importance of a fine regulation of the sperm energy metabolism in order to optimize sperm function.

Notwithstanding, seminal plasma must contain other components than those merely acting as motility activators. In this way, functions such as protection against immunological system of female genital tract and signaling to achieve total "in vivo" capacitation into the oviduct are also very important roles associated with seminal plasma. As in case of motility activation, each species will contain separate compounds in their seminal plasma in order to achieve these roles and, at this moment, this is a poorly understood investigation field. Another possible role for seminal plasma is as energy source for the first steps of spermatozoa after ejaculation. Thus, plasma seminal sugars could be a feasible energy source. However, it is difficult to understand why seminal plasma in all species does not contain glucose as their main energy source, since glucose is the most important energy-producing monosaccharide for all of mammalian tissues. Instead of this, seminal plasma contains other sugars, specially fructose, but also sorbitol and other [6, 28, 32, 33, 36, 42, 45], which are not as efficient as glucose as primary energy sources (see as an example in boar sperm [36]. Again, investigators can only speculate on this point. However, recent data from our laboratory seem to indicate that sugars could play a role of specific sperm function modulator besides their energy-fuelling role. This point would be developed in a more in depth manner when discussing external energy sources of sperm, although the possibility that seminal plasma monosaccharides play another role than that of energy sources can be seriously considered.

In any case, after ejaculation only a small percentage of ejaculated spermatozoa are able to leave the ejaculation placement and subsequently they reach oviduct after their uterine transit. Of course, energy requirements of sperm that are in course to the oviduct through uterus are totally different to those immediately after ejaculation. Freshly ejaculated spermatozoa require an energy metabolism in which energy was rapidly generated, in order to support the great amount of energy required by spermatozoa to activate for leaving the ejaculation point. In contrast, spermatozoa that have reached uterus do not require this fast and great energy consumption. In this way, their energy requirements would be much less great and imperative. This is especially important in those species, like pig [31], in which transport through uterus is mainly carried out by uterine peristaltic contractions rather by

the sperm motion by itself. This drop in energy requirements will be surely linked to a change in energy-fuelling pathways, although at this moment the changes suffered by energy metabolism during this step are not well known.

However, this is not the last change that mammalian sperm metabolism has to suffer in their life time. Once sperm reach oviduct, they rest in the oviductal crypts until their re-activation, following ovulation. This resting step is of the utmost importance, since at this point sperm, in tightly contact with oviductal cells, reach full capacitated status [14, 55]. Capacitation implies a myriad of functional and structural changes, like loss of cell membrane cholesterol, increase in tyrosine, serine and threonine phosphorylation levels of a wide array of separate proteins and intracellular calcium mobilization, whose full description is not possible in this chapter (see [52, 53] as reviews). Capacitation, however, has a great interest in the sense that its full achievement again implies new energy requirements to carry out processes like the increase in tyrosine phosphorylation of specific sperm proteins, such as pro-acrosin [13, 19]. This new requirements will imply again new changes in energy metabolism, which will be closely linked to the progressive changes that sperm function must suffer during this period.

Finally, remnant sperm will be loaded from the oviductal crypts in order to undergo oocyte penetration. In this period, capacitated sperm adopt a totally specific motility pattern known as hyperactivated motility, with separate characteristics depending on the studied species [51]. Once reached the oocyte, sperm have to penetrate it, launching a series of energy-consuming processes like adherence to oocyte zona pellucida and subsequent acrosome exocytosis [29]. Again, energy requirements will change when comparing with other sperm life-span steps. In this sense, acrosome exocytosis will need a fast and intense energy burst and, in fact, it has been described that progesterone-induced acrosome exocytosis in boar sperm that were previously subjected to "in vitro" capacitation is simultaneous to an intense and transitory increase in  $O_2$  consumption, which would correspond to transitory mitochondria activation [43].

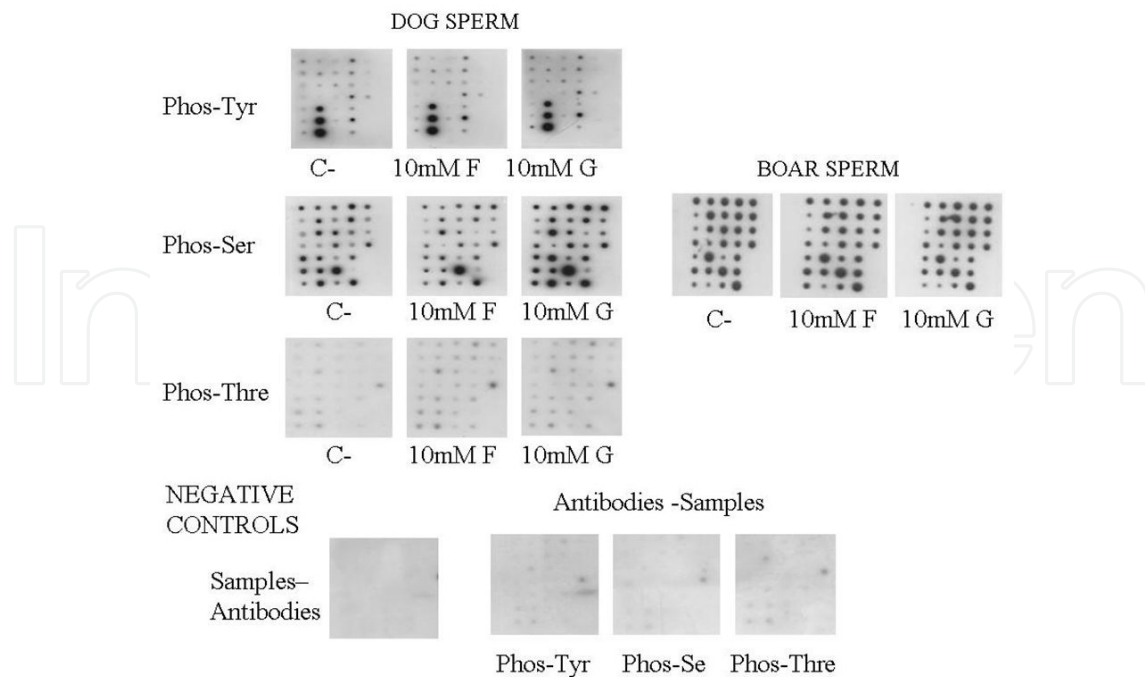
What are the main conclusions that can be yielded? The basic conclusion from all of this information would be that the dramatic changes that undergo mammalian spermatozoa from ejaculation to oocyte penetration must be accompanied by concomitant, dramatic changes in their energy regulating mechanisms. Little is known regarding how mammalian sperm modulate these changes, and this is one of the most challenging investigation fields that is currently open in the study of mammalian sperm function.

### **3. Energy sources for mammalian spermatozoa**

Energy production requires easy availability of energy substrates. In a general sense, any eukaryotic cell can obtain energy from either external or internal sources. External sources can be very different, from monosaccharides to lipids, whereas internal sources are mainly polysaccharides such as glycogen and lipids, although other internal sources can be aminoacids and other. Regarding mammalian sperm, separate external and also internal energy sources

have been described, offering thus a view not radically different to that observed in other eukaryotic cells. Notwithstanding, there are several characteristics that differentiates mammalian sperm to other eukaryotic cells in this point. Thus, the sequence of rapid location changes that underwent spermatozoa after ejaculation implies that they are synchronically placed in very separate locations inside female genital tract. This phenomenon will imply separate availability of external energy substrates, depending on the exact placement of spermatozoa. Moreover, the majority of cells integrated in a mammalian body will obtain their external energy sources directly from blood. This is not the case of spermatozoa, which are not keeping in direct contact with blood during their entire life. In this way, external energy sources might come from secretions of cells from the female genital tract or from seminal plasma. This implies that sperm must have very efficient mechanisms to uptake external energy resources, which will not be directly directed towards them. Moreover, and centering on seminal plasma as energy source, the time lapse that ejaculated sperm are in close contact with seminal plasma is, in fact, short. In fact, in species in which the ejaculated volume is short and rapidly placed inside a female genital tract of large size, like cow, contact between sperm and seminal plasma is very short, indeed. In any case, after ejaculation, both sperm and seminal plasma will immediately contact with secretions from the female genital tract. In this manner, sperm would be able to simultaneously find energy sources from both seminal plasma and the female genital tract. Only in species in which the volume of the ejaculate was very voluminous, like porcine, sperm will contact seminal plasma during a significant time lapse. Taking into account all of this information, it seems obvious that the adequate sperm external energy sources intake will depend of two main factors. The first factor will be the efficiency of external energy sources uptake mechanisms that sperm have developed. The second factor will be the specific mixture of external energy sources that sperm find in their journey inside the female genital tract. This mixture will come from both seminal plasma and female genital tract secretions, with predominance from one or the other source depending on the species and the exact location inside the genital tract.

All of this digression has been only centered in the origin of sperm external energy sources. However, what are exactly these energy sources? There is a general consensus in pointing at monosaccharides as the main energy sources for mammalian sperm [46]. Notwithstanding, sperm can utilize other substances than monosaccharides. Thus, boar sperm is able to utilize a wide range of substances, such as glycerol, lactate, pyruvate and citrate [26, 27, 37]. Other species are able to utilize also non-monosaccharide substrates as energy sources, although more information is needed to clarify this point (see as examples [22, 50]). The utilization of non-monosaccharide substrates as energy sources raises the question of the usefulness of these substrates. It has been suggested that these substrates could be an alternative in circumstances in which monosaccharide availability was limited [27, 37]. However, a thorough study of the energy substrates content that is present in each segment of the female genital tract is lacking, even in the best studied species. This impedes the complete elucidation of this suggestion. Despite this, the role of non-monosaccharide substrates as external energy sources for mammalian spermatozoa deserves a more in-depth study in order to clarify its biological importance and role.



**Figure 1.** Mini-array analysis of the tyrosine, serine and threonine phosphorylation status of several proteins involved in the regulation of cell cycle and overall cell function in dog and boar spermatozoa after incubation with glucose or fructose. Dog and boar spermatozoa were incubated for 5 min in the absence (C-) or presence of either 10mM fructose (10mM F) or 10mM glucose (10mM G). The tyrosine- (Phos-Tyr), serine- (Phos-Ser) and threonine-phosphorylation (Phos-Thre) levels of each spot in the mini-arrays were then analysed. The figure shows a representative image for five separate experiments. Figure excerpted from [17].

Turning on monosaccharides, one of the most intriguing questions is the ability of sperm to utilize a variety of sugars that are present, at least in seminal plasma. As indicated above, this is a very difficult question to study, since the sugars composition of seminal plasma is very different among species. In this sense, there are a significant number of species in which fructose is the main sugar, like human or mice [32]. However, in other species, like boar, fructose is not predominant [6, 32]. The main sugar in horse is not fructose or glucose, but sorbitol [42]. This sorbitol is further converted in fructose through the action of the enzyme sorbitol dehydrogenase, despite of old works indicating that horse sperm were not able to metabolize sorbitol [42]. Finally, there are also species, like dog, which lacks any monosaccharide in its seminal plasma [44, 48]. Another intriguing question is the mere presence of sugars, like fructose and sorbitol that are not typical in any other animal tissue. In fact, both fructose and sorbitol are typical vegetal sugars, without any significant presence in animals, excepting in mammalian seminal plasma. It is noteworthy that the utilization of sugars like fructose, sorbitol and mannose by sperm of species like boar and dog of is in fact less effective in order to obtain energy than that of glucose [36, 45]. The greater effectiveness of glucose is mainly linked to a greater sensitivity to the hexokinase system, which phosphorylates sugars as a first step in their metabolization pathway [36, 45]. Taking into account this lower efficiency, it is difficult to understand the biological logics to utilize sugars like fructose or sorbitol as mere energy sources for sperm. Another explanation for this apparent contradiction would be that non-glucose monosaccharides could exert other roles

than that of mere energy sources. In this sense, incubation of dog sperm with fructose induced a specific decrease of serine phosphorylation levels of several proteins with key roles in the regulation of sperm cell function, such as protein kinases Akt, PI3 kinase, ERK-1 and protein kinase C ([17] and Figure 1). Strikingly, the incubation with glucose induced a specific increase in the serine phosphorylation levels of other key regulatory proteins, like c-kit, Raf-1, tyrosine kinase and several protein phosphatases ([17] and Figure 1) These effects might induce sugar-specific changes in the overall dog sperm function. On the contrary, the incubation of boar sperm with either glucose or fructose did not induce any of the specific actions observed in dog sperm ([17] and Figure 1). All of these results clearly indicate that sugars can have a sugar- and species-specific action as signaling compounds, modulating thus sperm function in a closely-linked manner, depending on the moment in which sperm and sugar were kept in contact. In this way, the idea of seminal plasma sugars as mere energy sources would be dismissed and being substituted by another idea; sugars as both energy sources and direct sperm function modulators in a simultaneous and coordinated form.

External sources, however, are not the only possibility found by mammalian sperm to obtain energy. Sperm can obtain energy also from endogenous sources. One of the most studied sources is glycogen. The presence of a functional glycogen metabolism has been demonstrated in several species, such as dog, boar, horse, ram and bonnet monkey [5], although no glycogen was found in other species like mice and rat [3]. The primary role of these internal energy sources would be, logically, the maintenance of a limited energy reservoir, although this could not be a universal function. In fact, dog sperm glycogen plays a role in the achievement of "in vitro" capacitation in a medium without glucose by being a key intermediate metabolite in the obtainment of energy through gluconeogenesis, which was essential to the achievement of the capacitated status [1, 2]. Thus, in dog sperm, glycogen plays an important role as capacitation regulator, besides its energy reservoir role. In this manner, a more in-depth study of the exact role of glycogen should be needed to obtain a clearer picture of the utilization of endogenous substrates as energy sources in sperm.

#### **4. Main metabolic pathways to obtain energy and control mechanisms involved in the coordination of energy management of mammalian sperm**

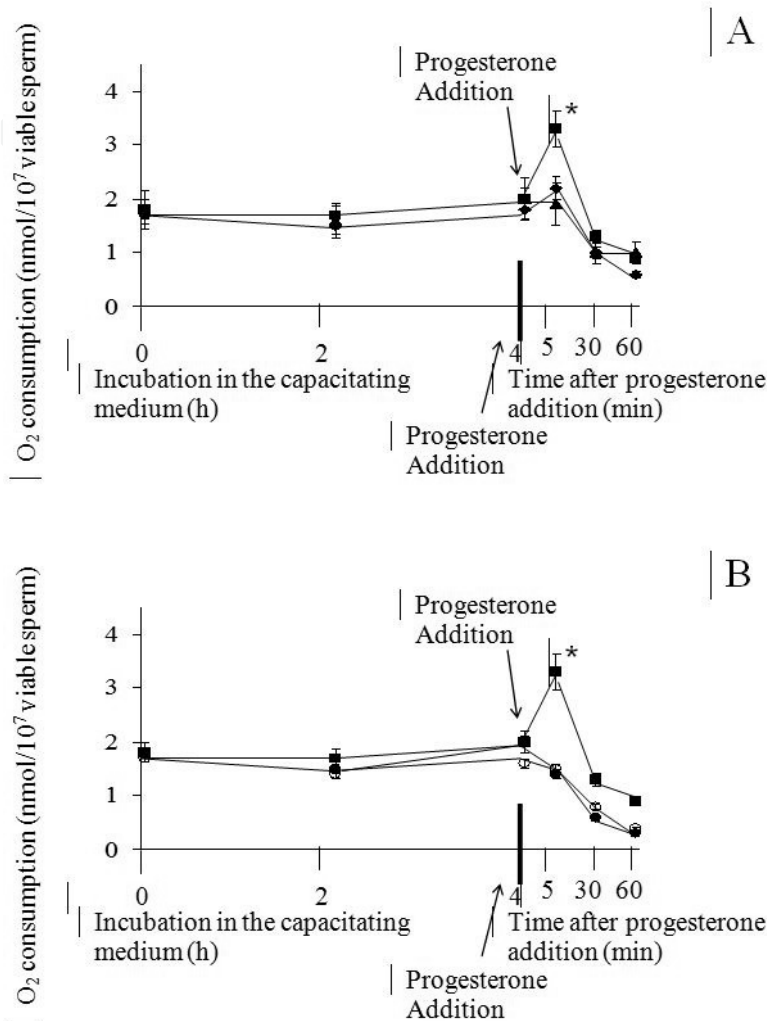
If monosaccharides are considered as the most important exogenous energy source for mammalian sperm, the question of the precise metabolic pathways by which mature mammalian sperm obtain energy is relatively straightforward. Thus, the main metabolic pathway is glycolysis. The preeminence of glycolysis in freshly ejaculated sperm has been demonstrated in species like bull, mice and boar [21, 33, 39]. In fact, in species like boar, at least the 95% of the energy obtained from glucose is obtained through the glycolytic pathway in freshly obtained ejaculates [33]. The explanation of the glycolysis preeminence in these species is easy to understand. Glycolysis would reach high velocity rates taking from a very high velocity of sugars intake and phosphorylation. This will be due to the very high sensi-



tivity to sugars of both GLUTs monosaccharide transporters and overall hexokinase activity. In fact, glycolytic rate is so high in species like bull that it rarely achieve the theoretical stoichiometric ATP yield of the glycolytic pathway, living thus to the establishment of an active substrate cycling, important to the maintenance of motility [21]. In fact, the ability of generate energy of a specific sugar would depend on the ability of each sugar to be uptaken and subsequent phosphorylated. In this way, it is important to remind that this sensitivity changes upon a sugar- and species-specific basis. Thus, as described above and taking porcine sperm as a basis, it is important to remind that the velocity by which glucose is phosphorylated and then incorporated to the glycolytic flux is greater than that observed by other sugars like fructose, sorbitol and mannose [36]. The ability of each species to utilize each separate sugar will be then different, depending on the specific machinery that sperm have in order to uptake and further phosphorylate monosaccharides. In fact, this machinery can be more different among species than that previously thought. As an example, dog sperm have two separate hexokinase activities. The first has a very high sensitivity for sugars as glucose, with a  $K_m$  of about 0.1 mM. The second hexokinase activity has much lower glucose sensitivity, with kinetic properties very similar to those described for hepatic glucokinase [16]. This sophisticated machinery makes dog sperm able to develop a dual reactivity to react against very separate glucose concentrations, specifically changing sperm function in contact with environments with these separate characteristics. Remarkably, sperm from other species such as boar have not any glucokinase-like activity [16], reflecting thus a species-specific reactivity against glucose that is initiated at the very start of the glucose utilization pathway.

The final sugar utilization step is the entry of pyruvate obtained at the end of glycolysis into mitochondria to be subsequent degraded into the mitochondrial respiration system. There is not a universal agreement regarding the importance of mitochondria-based energy obtainment. In this way, an optimal mitochondrial function has been related not only with sperm motility in bull [18], horse [20], ram [34] and mouse [39] but also with fertilization ability in human (30). However, gene knock-out of the glycolytic enzyme glyceraldehyde-phosphate dehydrogenase (GAPDH) in transgenic mice caused the appearance of non-motile sperm and a significant reduction of the ATP content (10% of the total) despite having no deficiency in oxygen consumption [38]. This seems to imply that although a correct mitochondrial function is needed to the maintenance of an optimal sperm function, mitochondrial respiration would not be the most important role of mitochondria to exert their activity. Another explanation would be that mitochondrial respiration would not be important in the maintenance of the overall energy status of sperm, although it would be of the utmost importance in the maintenance of punctual aspects of sperm function. In this sense, progesterone-induced acrosome exocytosis of boar sperm subjected to a previous "in vitro" capacitation is concomitant with a rapid, intense and transitory burst of oxygen consumption [42]. Moreover, unpublished results from our laboratory show that the inhibition of this oxygen consumption burst is concomitant with an almost complete lack of progesterone-induced acrosome exocytosis (Figure 2 and data not shown). These results are concomitant with overall low levels of oxygen consumption, which in fact indicate that the majority of ATPs obtained by boar sperm do not come from mitochondrial respiration [33]. However, the re-

sults seem to indicate that the minority mitochondrial respiration is essential to obtain a feasible progesterone-induced acrosome exocytosis.



**Figure 2.** Rhythm of O<sub>2</sub> consumption of boar sperm subjected to “in vitro” capacitation and subsequent “in vitro” acrosome reaction in the presence or absence of oligomycin A or in Ca<sup>2+</sup>-depleted capacitation medium. Boar sperm were incubated for 4h and then were added with 10 µg/mL progesterone and subjected to a further incubation for 60 min. A): Sperm cells incubated in a standard capacitation medium or in media added with 2.4 µM oligomycin A. ■: Control cells. ◆: Spermatozoa incubated in capacitation medium added with 2.4 µM oligomycin A from the beginning of the incubation. ▲: Spermatozoa incubated in a standard capacitation medium for 4h and subsequent added with 10 µg/mL progesterone and 2.4 µM oligomycin A together. B): Sperm cells incubated in a standard capacitation medium or in Ca<sup>2+</sup>-depleted media. ○: Spermatozoa incubated in capacitation medium without Ca<sup>2+</sup> and added with 2 mM EGTA from the beginning of the experiments. ●: Spermatozoa incubated in a standard capacitation medium for 4h and subsequent added with 10 µg/mL progesterone and 2 mM EGTA together. Results are expressed as means ±S.E.M. for 7 separate experiments. Asterisks indicate significant (P<0.05) differences when compared with the respective Control values. Results excerpted from [43]) and unpublished data from our laboratory.

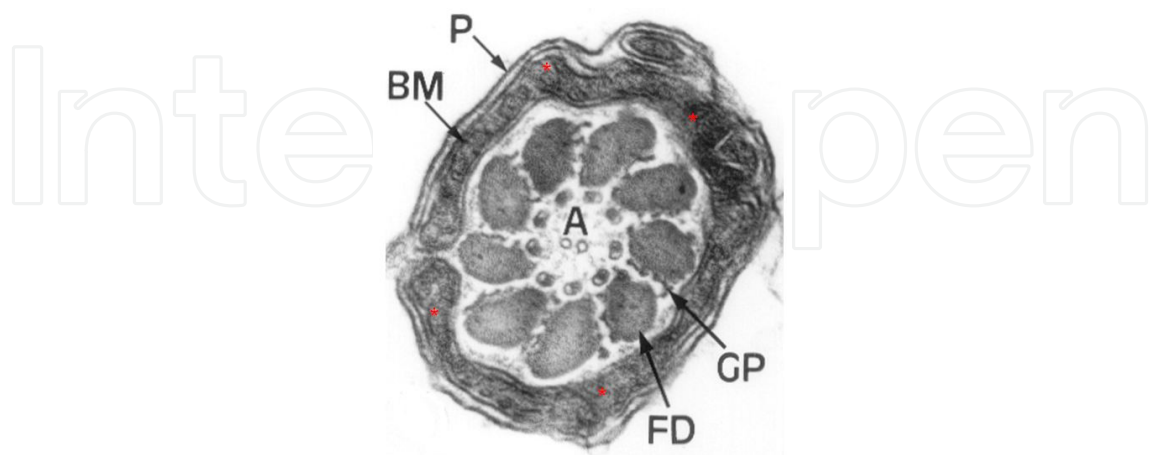
However, as exposed above, monosaccharides are not the only energy source that sperm can utilize. Other substrates, such as citrate and lactate, can be utilized to obtain energy, at least in several mammalian species. The ways by which mammalian sperm utilize these non-

monosaccharide substrates have not been as thoroughly studied as those linked to monosaccharide metabolism. In this way, boar sperm have been one of the most studied species. In this species, extracellular citrate and lactate are utilized after their intake by metabolism through the Krebs cycle [37]. This metabolism is the same than that detailed for many other cellular types. However, sperm utilization of citrate and lactate has several specific features. Thus, a sperm-specific lactate dehydrogenase (LDH) isozyme has been described in several species [12, 27, 37, 40, 41]. This specific isozyme, named LDH-X is the most important LDH form in sperm in which it has been described, such as boar [27], whereas its activity presents several differentiate features. In this sense, the LDH-X is distributed in both soluble and non-soluble fractions of sperm extracts obtained through sonication [37], indicating thus the existence of a specific distribution pattern of this LDH-X in sperm. Moreover, the kinetic characteristics of the LDH are different, depending on the location of the enzyme, either in the soluble or the non-soluble sperm extract fraction [37]. In fact, immunocytochemistry of boar sperm has shown that the LDH-X is mainly located at the mid-piece and principal area of the tail, linking thus its activity to the neighboring of mitochondria-located Krebs cycle activity [37]. All of these information clearly indicate that the regulation of sperm LDH activity, and hence lactate metabolism, is regulated in a very complex manner, with mechanisms depending on factors such as the precise location of the key regulatory enzymes. Another interesting feature of both sperm lactate and citrate metabolism is that lactate enters the Krebs cycle through a direct pathway, which does not need its previous conversion to pyruvate [27, 37]. This direct pathway is important, since it not only produces energy, but also relevant levels of reductive potential, allowing sperm to regenerate significant amounts of  $\text{NAD}^+$ . Regarding citrate, sperm can metabolize it through two simultaneous pathways. The first pathway is through direct utilization by Krebs cycle, yielding  $\text{CO}_2$  and ATP. The second pathway is indirect, by following two sequential steps. A first step in which citrate enters into the Krebs cycle. In the second step the metabolites derived from citrate after its pass through the Krebs cycle are directed to the pyruvate carboxylase step, which converted these metabolites in lactate, which, in turn, will be sent to the extracellular medium and again re-entered into the Krebs cycle through the LDH-X step. At first glance, the biological meaning of this second, convoluted pathway is not immediately understood. However, if the maintenance of a correct  $\text{NAD}^+/\text{NADH}$  equilibrium is considered as basic to maintain a proper sperm function, the main objective of this second, indirect pathway would be not the obtainment of energy, but of reductive potential. In this way, citrate and lactate can have a paramount role not as energy producers, but as reductive potential metabolites.

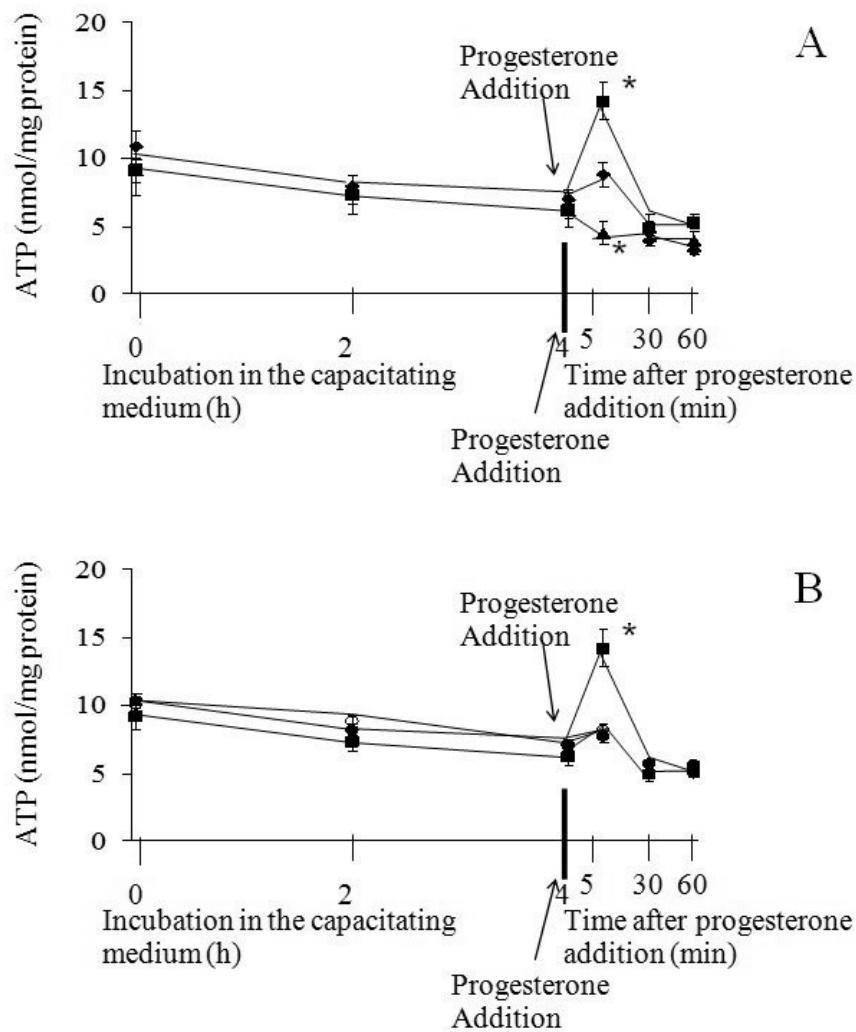
## 5. Roles of mitochondria in the control of the overall mature boar sperm function

As previously indicated, the main energy source for mature mammalian sperm are ATPs obtained either through the glycolytic pathway or mitochondrial oxidative pathways. The precise equilibrium between both energy-obtaining pathways will be different among species and in cases like boar and mice, this equilibrium is greatly unbalanced towards glycolysis, which is the overly majoritary energy-obtaining pathway in the presence of sugars like

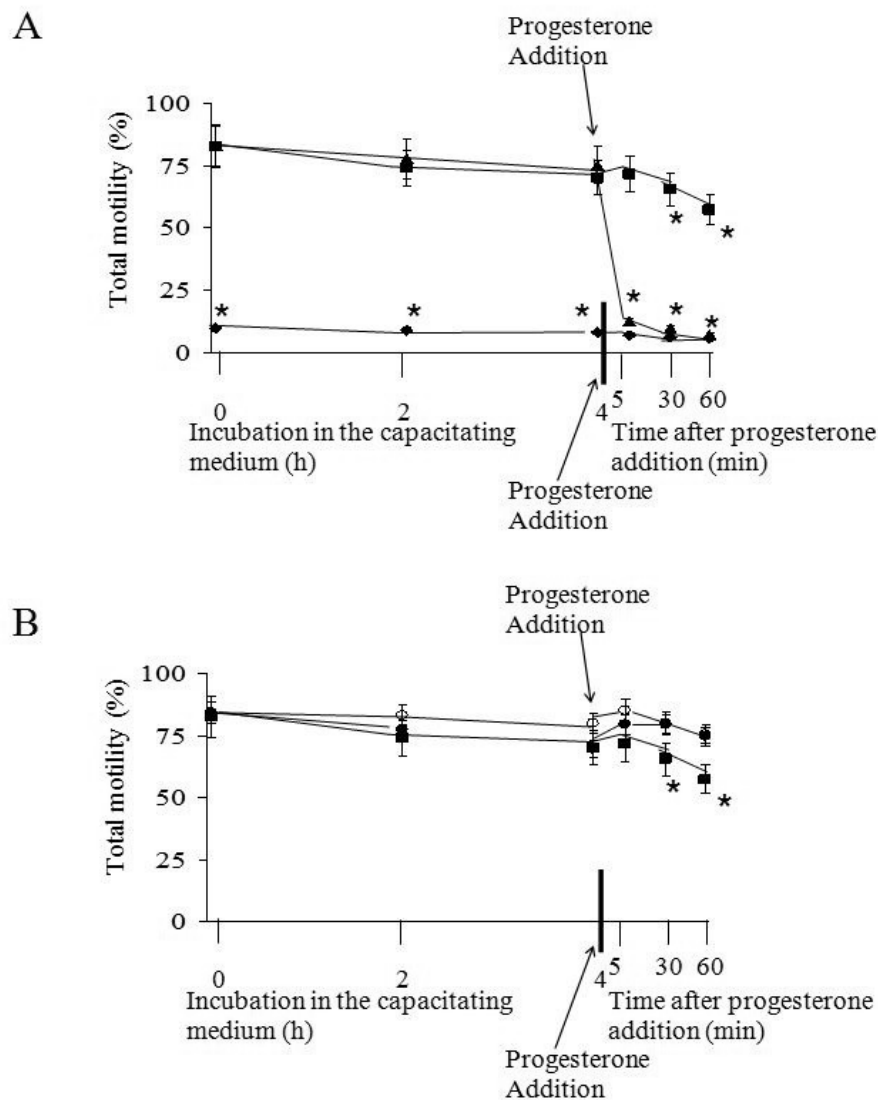
glucose [33, 39)]. This pre-eminence of glycolysis in species like boar and mouse arises to an important question, if sperm mitochondria seem not to have a predominant role in these species in obtaining energy, what are their main role? Investigators can only speculate on this point, although there are several data regarding mainly boar sperm that can aid to obtain a better vision of this issue. The first data correspond to the observation of boar sperm mitochondria ultra-structure (Figure 3). Electron microscope images of boar sperm mitochondria show an organelle with very few prominent inner membrane crests. Instead of this, the inner mitochondrial space is mainly occupied by thin and short crests and with an amorphous and homogeneous matrix. This is very different to the classical image for mitochondria, which, like those from hepatocytes, show an inner structure crowded with prominent inner crests. Taking into account that the most important steps of the electronic transport system and subsequent ATP synthesis are structurally linked to inner mitochondrial crests, it is easy to assume that boar sperm mitochondria would not be very efficient as energy suppliers. In fact, the oxygen consumption rate of boar sperm, which is a direct measure of mitochondrial ability to generate energy, is about 2 magnitude orders lower than that measured in pig hepatocytes [4, 43]. However, this does not preclude that mitochondria-originated energy would not be important for sperm function in species in which glycolysis is the most important energy-synthesizing pathway. Regarding this point, our laboratory has shown that the achievement of a feasible, progesterone-induced “in vitro” acrosome reaction is concomitant with a sudden and intense peak of O<sub>2</sub> consumption rate and also of intracellular ATP levels ([43] and Figures 2,4). Furthermore, unpublished data from our laboratory clearly shows that this peak is not present in conditions in which progesterone-induced acrosome reaction is prevented. These results strongly suggest the existence of a close relationship between mitochondria-generated energy and the achievement of the acrosome reaction, despite of the low energy-efficiency of these organelles.



**Figure 3.** Ultrastructural image of boar sperm mitochondria. The low development of inner crests is noticeable (asterisks). BM: inner mitochondrial membrane. P: cell membrane. A: axoneme. FD: dense fibres. GP: peripheral granules. From [7]).



**Figure 4.** Intracellular ATP levels of boar sperm subjected to “in vitro” capacitation and subsequent “in vitro” acrosome reaction in the presence or absence of oligomycin A or in Ca<sup>2+</sup>-depleted capacitation medium. Boar sperm were incubated for 4h and then were added with 10 µg/mL progesterone and subjected to a further incubation for 60 min. A): Sperm cells incubated in a standard capacitation medium or in media added with 2.4 µM oligomycin A. ■: Control cells. ◆: Spermatozoa incubated in capacitation medium added with 2.4 µM oligomycin A from the beginning of the incubation. ▲: Spermatozoa incubated in a standard capacitation medium for 4h and subsequent added with 10 µg/mL progesterone and 2.4 µM oligomycin A together. B): Sperm cells incubated in a standard capacitation medium or in Ca<sup>2+</sup>-depleted media. ○: Spermatozoa incubated in capacitation medium without Ca<sup>2+</sup> and added with 2 mM EGTA from the beginning of the experiments. ●: Spermatozoa incubated in a standard capacitation medium for 4h and subsequent added with 10 µg/mL progesterone and 2 mM EGTA together. Results are expressed as means±S.E.M. for 7 separate experiments. Asterisks indicate significant (P<0.05) differences when compared with the respective Control values. Results excerpted from unpublished data from our laboratory.



**Figure 5.** Percentages of total motility of boar sperm subjected to “in vitro” capacitation and subsequent “in vitro” acrosome reaction in the presence or absence of oligomycin A or in Ca<sup>2+</sup>-depleted capacitation medium. Boar sperm were incubated for 4h and then were added with 10 µg/mL progesterone and subjected to a further incubation for 60 min. Total motility has been defined as the percentage of spermatozoa with a curvilinear velocity (VCL) higher than 20 µm/sec. A): Sperm cells incubated in a standard capacitation medium or in media added with 2.4 µM oligomycin A. ■: Control cells. ◆: Spermatozoa incubated in capacitation medium added with 2.4 µM oligomycin A from the beginning of the incubation. ▲: Spermatozoa incubated in a standard capacitation medium for 4h and subsequent added with 10 µg/mL progesterone and 2.4 µM oligomycin A together. B): Sperm cells incubated in a standard capacitation medium or in Ca<sup>2+</sup>-depleted media. ○: Spermatozoa incubated in capacitation medium without Ca<sup>2+</sup> and added with 2 mM EGTA from the beginning of the experiments. ●: Spermatozoa incubated in a standard capacitation medium for 4h and subsequent added with 10 µg/mL progesterone and 2 mM EGTA together. Results are expressed as means±S.E.M. for 7 separate experiments. Asterisks indicate significant (P<0.05) differences when compared with the respective Control values. Results excerpted from (42) and unpublished data from our laboratory

However, the fact that Krebs cycle seems to be important only in punctual moments of the boar sperm life-time does not necessarily indicate that boar sperm mitochondria are only important in this point. It is noteworthy that mitochondria have much more roles than purely

being a mere energy-producing factory. Mitochondria also play a key role in the control of other very important aspects of eukaryotic cells function, like modulation of apoptosis and the control of calcium metabolism. Thus, it is very probable that mitochondria from sperm of species like boar or mouse exert their most important functions on other cellular functional points than energy management. Unpublished results from our laboratory are strongly pointing out this supposition. Thus, the incubation of boar sperm in a capacitation medium in the presence of oligomycin A, a specific inhibitor of the electronic chain and the chemiosmosis steps [11], immobilizes boar sperm and prevent them to achieve "in vitro" capacitation. However, this effect was accomplished without any significant changes in the rhythm of  $O_2$  production and the intracellular ATP levels (Figures 2, 4, 5 and data not shown from our laboratory). In contrast, the incubation of boar sperm in a capacitation medium without calcium induces an increase in the velocity parameters of these cells, although the achievement of capacitation is also prevented (data not shown). The effect linked to the lack of extracellular calcium however, is again concomitant with no changes in both the rhythm of  $O_2$  production and the intracellular ATP levels (Figures 2, 4 and data not shown). The conclusion from these results is that boar (and probably mice) sperm mitochondria play an important regulatory role in the control of functional aspects such as motility patterns and the achievement of "in vitro" capacitation by ways that are not directly linked to energy production. This opens a new perspective in the manner in which investigators would have to approximate to the understanding of the mitochondria role in the control of sperm function. However, much more work is needed in order to achieve a complete view of this complex phenomenon.

## **6. Metabolic phenotypes: a result of the separate evolutionary strategies developed by mammals to optimize reproductive indexes**

All of data showed above highlight a phenomenon that has not been much explained, although it is well known by all of investigators in this field. This phenomenon is the strong species-specificity that energy obtainment mechanisms show when comparing separate mammals. Differences are so intense that several metabolic phenotypes can be defined, depending on the metabolic characteristics showed by each species. In this manner, there are at least two separate metabolic phenotypes regarding mammalian spermatozoa. The first phenotype will be composed by species in which energy substrates, mainly monosaccharides, will be directed to the practically immediate utilization of all of the assimilated sugars through the appropriate catabolic pathways, especially glycolysis. This specific metabolic phenotype is very common in mammalian sperm, especially in those species, which do not require a long, sperm-survival time-lapse inside the female genital tract such as pig and bull [24, 47]. However, a second phenotype is evident in species where sperm survival inside the female genital tract must be relatively long, such as the dog [15]. In these species, an energy strategy based upon an entirely catabolic metabolism would not be efficient. The optimization of energy Management in relatively long-living sperm like dog would be optimized with the presence of alternative anabolic pathways, such as glycogen synthesis, which allows for the maintenance of a significant mid-to-long intracellular energy reserve. This re-

serve would play an important role in the maintenance of “in vivo” sperm survival. In fact, as discussed above, the existence of a fully functional glycogen metabolism has been demonstrated in sperm from species like dog, boar, horse and ram [5]. Remarkably, dog sperm shows the most active glycogen metabolism of all of the studied species, in this way accumulating the maximal recorded intracellular levels [5]. As described above, this glycogen plays an important role in the achievement of feasible “in vitro” capacitation [1, 2], reinforcing thus the importance of this anabolic pathway in dog. The importance of glycogen synthesis in dog would be surely linked to another important feature, also described above. It is worth noting that dog sperm is the only studied species so far that shows the presence of two separate hexokinase activities. The first of them is similar to hexokinase-I, which is present in all of the studied mammalian sperm. The second, however, is similar in kinetic and immunologic properties to the hepatic and pancreatic isoform glucokinase [16]. The presence of a glucokinase-like activity in dog sperm but not in other species like boar acquires utmost importance when the precise role that hepatic and pancreatic glucokinase plays is studied. Thus, it is well known that hepatic glucokinase acts as a “metabolistate” that diverts hexoses metabolism to either anabolic or catabolic pathways, depending on factors such as the precise physiologic cell status and sugar extracellular levels [9]. If a similar role for the dog sperm glucokinase-like activity is assumed, the inference that this protein also regulates the entry of energy metabolites in either anabolic or catabolic pathways can be also yielded. These assumptions, notwithstanding will depend on both the precise energy necessities and the extracellular concentrations of sugars inside the female genital tract. Moreover, this “metabolistate” seems to be in the basis of above described, observed differential effects of fructose and glucose in the serine phosphorylation levels of dog sperm proteins like protein kinase C [17]. Thus, dog sperm reaches an even more fine regulation of not only their intracellular energy levels, but also their overall functional status. This very fine regulation would surely increase survival ability of these cells.

These two separate metabolic phenotypes would not surely be the only present among mammalian species. Much more work is needed in order to describe and analyze this phenomenon. In any case, the existence of these metabolic phenotypes would be of the greatest importance. These phenotypes, in fact, will be the reflection of the sperm specialization due to the adoption of separate reproductive strategies among mammals. Thus, these separate evolutive, reproductive strategies will cause the existence of great differences among sperm of separate species not only under a morphological, but also under a metabolic point of view. These differences among species would be, in turn, at the basis of the described differences in vital aspects of sperm function, such as motility patterns and capacitation mechanisms. Finally, these physiological differences would also be reflected in changes in the specific strategy developed to store a particular semen sample from a precise species in optimal conditions.



## 7. Modulation of energy metabolism as a tool to improve IA results

It seems obvious that a good regulation of the energy regulation mechanisms would be of the utmost importance in order to optimize sperm storage and, hence AI results. Surprisingly, very few investigations have been conducted on this specific point. This could be due to the historical misinterpretation of sperm energy regulatory mechanisms. Historically, these mechanisms has be considered as being simple and linear and, hence of little practical importance [32]. In this way, the majority of semen extenders contain inordinate concentrations of various sugars, like glucose and fructose. The basis for this addition is the thinking that sperm will utilize separate sugars in a similar manner and by linear, concentration-dependent mechanisms. This strategy has at least three weak points. The first point is the fact that the optimal utilization of sugars by sperm is reached to determined concentrations of this sugars. For instance, the optimal utilization rate of glucose by sperm from dog and boar is reached to very low concentrations of the sugar, at about 0.1 mM [16, 36, 37, 45]. This indicates that the addition of low concentrations of sugar to the extenders would be enough to maintain sperm energy levels. This is not followed by the majority of extenders, in which sugars are added to concentrations above 50 mM. At these concentrations, the sperm energy machinery is overrated and non-optimal, despite the fact that cells are stored to low temperatures. The second weak point is the fact that, as described above, sugars could have other effects that being mere energy supplies (see [17]). In this case, the election of either glucose or fructose in a species can influence their ability of survival by modifying specific aspects of sperm functionality. The third weak point is that mammalian sperm are abler to utilize non-glucidic substrates as energy sources. Nonglucidic substrates like lactate and citrate are frequently added to semen extenders in order to play roles that are not related to the maintenance of sperm energy levels. Some of these roles are, for instance, maintenance of osmolarity and pH. However, sperm cells can consume these substances and, in this way, the extender design would lose its conservative properties, since some protective functions (maintenance of osmolarity, pH, etc.) could be impaired when these substances are metabolized by sperm. Following this rationale, the exact proportion of glucose and nonglucidic substrates like citrate and lactate greatly affects several parameters of boar-semen quality analysis during storage at 15°C-17°C. Some of the parameters affected by the exact sugar/non-sugar composition of extenders were the membrane integrity, the response to functional tests like the osmotic resistance test and the overall mid-term survival at 15°C-17°C [35]. These results strongly suggest that the exact proportion of these substrates, more than their final concentration, is of the greatest importance to optimize the maintenance of sperm function during sperm storage in refrigerated conditions.

As a conclusion, the lack of a proper knowledge of the mechanisms linked to the control of mammalian sperm energy management is hampered a further optimization of the semen extenders utilized in the different species. This would have a detrimental effect in the subsequent AI results obtained with semen stored in sub-optimally designed extenders. This highlights the great interest in more investigations in order to elucidate the exact mechanisms of energy management in all of the domestic mammalian species.

## 8. Conclusion

Energy management of mature mammalian spermatozoa is a much complex question than that usually devised. This complexity is due to a combination of factors, such as the existence of rapid and profound environmental changes during the entire life of sperm post-ejaculation, as well as the development of many different evolutionary reproductive strategies among mammalian species, which lead sperm to develop specific energetic strategies. In this sense, factors like the time that sperm have to spend inside the female genital tract or the existence of competence among sperm from separate males inside the female will play important roles in the design of an optimal energy management strategy in each mammalian species.

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