

## EFFECT OF SOME IONS ON SPERM ACTIVATION IN *Brycon henni* (EIGENMANN 1913)

### Efecto de algunos iones sobre la activación espermática en *Brycon henni* (Eigenmann 1913)

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#### ABSTRACT

Spermatozoa in Characid fish remain immobile in seminal plasma and are activated when freed into water where the ionic balance seems to be the main factor starting the activation process. This process was the target of the present study with emphasis on the activation of motility and on motility maintenance over time. The effect of isosmotic solutions was analyzed taking into account the possible combinations of the following ions, Ca<sup>2+</sup>, K<sup>+</sup>, Mg<sup>2+</sup> and Na<sup>+</sup> as well as the effect of channel blocking agents. The parameters measured were cells with motility (%), duration of motility (s), plasma membrane potential, and the effect of channel blockers on activation time and on motility. There was an increased motility when the semen was incubated in solutions containing K<sup>+</sup> (p<0.05) compared with the control (CaNaMgK solution); the longest duration of motility was attained when the incubation was performed in solutions containing Na<sup>+</sup> and Mg<sup>2+</sup> (p<0.05). All solutions induced a change in membrane potential detected after 15 s of activation. Blocking K<sup>+</sup>, Ca<sup>2+</sup> and Na<sup>+</sup> channels did not alter motility but decreased the activation time (p<0.05). Potassium induced activation at all concentrations up to 105 mM, but motility was drastically decreased at concentrations higher than 140 mM (p<0.05). The conclusion is that interaction of the ionic environment with the cell membrane leads to changes in membrane potential and intracellular signalling that trigger sperm motility in *Brycon henni*.

**Key words:** Ionic balance, channel blockers, membrane potential, Oxonol.

#### RESUMEN

Los espermatozoides en los carácidos se mantienen inmóviles en plasma seminal, activándose cuando se liberan en el agua. El objetivo de este estudio fue conocer algunos de los mecanismos que inducen la activación espermática (porcentaje de espermato-

zoides que adquieren movilidad y el tiempo que dura la misma). El efecto de las soluciones isosmóticas, se analizó considerando las combinaciones de los iones  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$  y  $\text{Na}^+$ , y el efecto de algunos bloqueadores de canales iónicos. Se evaluó el porcentaje y duración de la movilidad, así como el potencial de la membrana plasmática. La motilidad espermática fue mayor cuando el semen se incubó en las soluciones que contenían  $\text{K}^+$  ( $p < 0,05$ ) comparado con el control positivo. La mayor duración de la movilidad se obtuvo cuando se incubaron los espermatozoides en soluciones que contenían  $\text{Na}^+$  y  $\text{Mg}^{2+}$  ( $p < 0,05$ ). Todas las soluciones probadas indujeron un cambio en el potencial de membrana, detectado después de 15 segundos de la activación. Bloqueo de los canales de  $\text{K}^+$ ,  $\text{Ca}^{2+}$  y  $\text{Na}^+$  no alteró la movilidad, sino que disminuyó el tiempo de activación ( $p < 0,05$ ). Los iones potasio indujeron la activación en todas las concentraciones hasta 105 mM, pero la movilidad disminuyó drásticamente cuando las concentraciones fueron mayores de 140 mM ( $p < 0,05$ ). Se concluye que la interacción del ambiente iónico con la membrana de la célula conduce a cambios en el potencial de la membrana que conllevan a señales intracelulares que desencadenan la movilidad del esperma.

**Palabras clave:** balance iónico, bloqueadores de canales, potencial de membrana, Oxonol

## INTRODUCTION

In Colombia the fish *Brycon henni* is a protected endemic species. It inhabits water bodies in coffee producing areas (700-1900 m.a.s.l.; 4°35'56"N - 74°04'51"W; 18-28°C). Insufficient knowledge of its basic biology and behavior prevent the commercial culture of this promising fish. Our interest is focused in sperm physiology of captive males, in order to improve fish biotechnologies.

Ionic balance in seminal plasma is important for the maturation of sperm (Ingerman *et al.*, 2002), to keep spermatozoa immotile within the testis and in the spermatic duct (Müller *et al.*, 1994), to develop the capacity of spermatozoa to move (Mochida *et al.*, 1999; Ohta *et al.*, 2001), and to maintain adequate motility when the sperm is delivered into the water (Morisawa and Suzuki, 1980; Scott and Baynes, 1980; Márián *et al.*, 1993; Cosson *et al.*, 1999). Activation of sperm and its relationship with ions has been demonstrated by inducing an osmotic shock (Krasznai *et al.*, 1995; Márián *et al.*, 1997), by changing the transmembrane potential (Emri *et al.*, 1998), and through the regulation of interchangers and ionic channels for  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  (Krasznai *et al.*, 1995; Cosson *et al.*, 1999; Vines *et al.*, 2002; Krasznai *et al.*, 2003; Felix *et al.*, 2004). This ionic balance is believed to be related, among other factors, to the intracellular environment (Krasznai *et al.*, 1995; Márián *et al.*, 1997), the composition of the seminal plasma (Mochida *et al.*, 1999; Ohta *et al.*, 2001) and the ionic flow through the membrane that in turn is regulated by channels. Several types of channels have been described: 1) open pore channels: that function by gradients (Felix *et al.*, 2004), 2) gate channels activated mechanically that open when the membrane expands due to turgency (Cosson *et al.*, 1999; Krasznai *et al.*, 2003), 3) voltage-dependent channels that are activated when there is a change in membrane potential (Krasznai *et al.*, 2000; Felix *et al.*, 2004), 4)

ligand channels working through specific proteins that interact with their receptors (Felix *et al.*, 2004), and finally 5) interchanger channels in which the energy generated by the passage-involving an ion gradient-through the channel is used for the passage of another ion in the opposite direction (Vines *et al.*, 2002). Mitochondria, having channels in both the internal and the external membranes, also regulate the intracellular ionic balance (Garlid and Paucek, 2003) allowing the flux of  $\text{Ca}^{2+}$ ,  $\text{K}^+$  and  $\text{Mg}^{2+}$  in the cell to flow in either direction (entry-exit).

Cytosolic  $\text{Ca}^{2+}$  is a second messenger and a cofactor in the activation process: it activates adenylyl cyclase to induce the production of cyclic AMP necessary for phosphorylation of tubulin and then for flagellar axoneme activation (Eddy and O'Brien, 1994; Suarez and Ho, 2003).  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  are necessary to produce the ATP required for oxidative phosphorylation (Sordahl and Silver, 1975; Barbiroli *et al.*, 1999) that depends directly on the membrane potential and on the presence of metabolic precursors such as glucose (Lahnsteiner *et al.*, 1998). The dinein ATPase of the axoneme utilizes ATP to bring about movement of the flagellum (Suarez and Ho, 2003). In teleosts, spermatozoa become activated when they are in contact with water: in the sea due to a hyperosmotic shock (Vines *et al.*, 2002), and in fresh water due to a hyposmotic shock (Morisawa *et al.*, 1983). The objective of this study was to expand our knowledge on the role of ions on the inhibition and activation of sperm function in *Brycon henni*.

## MATERIALS AND METHODS

**FISHES AND SAMPLES.** Average fish body weight and size (total length) were  $112 \pm 7$  g and  $20.5 \pm 0.7$  cm respectively. Semen was collected by craneo-caudal massage during the season of maximal sperm production from fish kept in captivity for one year. The samples were collected in 1.5 ml tubes and maintained at  $4^{\circ}\text{C}$  for 5-7 h until used. Five sexual mature male fish were used, and four samples were collected in different dates during the reproductive season; thus, a total of twenty samples were tested with the ionic solutions (see next) at a 1:1000 dilution. The average semen concentration used with the various solutions was  $2.3 \pm 1.10$  spermatozooids/ml.

**IONIC SOLUTIONS.** Previous seminal plasma characterization was taken into account (Tabares *et al.*, 2006). Fifteen different combinations of isosmotic solutions were prepared with  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$  and/or  $\text{Na}^+$  (KCl: 37.3 mM, NaCl: 122.8 mM,  $\text{CaCl}_2$ : 0.87 mM,  $\text{MgCl}_2$ : 0.83 mM (see Fig. 1). Mannitol was used to replace the missing ion(s) to keep osmolality at 307 mOs/kg. The positive control was a solution with an ion concentration similar to that of seminal plasma (KCl: 37.3 mM, NaCl: 122.8 mM,  $\text{CaCl}_2$ : 0.87 mM,  $\text{MgCl}_2$ : 0.83 mM (Tabares *et al.*, 2006). The negative control was an isosmotic solution of mannitol (63.63 mM). All solutions were buffered with 20 mM Hepes (pH 7.5).

**EFFECT OF INCUBATION IN IONIC SOLUTIONS ON SPERM MOTILITY AND ACTIVATION TIME.** To determine the moment at which activation started and the activation time, the semen was diluted 1:1000 in each of the 15 solutions to be tested. The tubes were incubated for one hour at  $20^{\circ}\text{C}$  and activated with a hyposmotic water solution

containing 20 mM Hepes (pH 7.5; activator solution) at 20 mOs/kg. The gross motility and the activation time (in seconds) were measured (Tabares *et al.*, 2006) using an inverted phase contrast microscope (Nikon TMS-F 211571) at 40X.

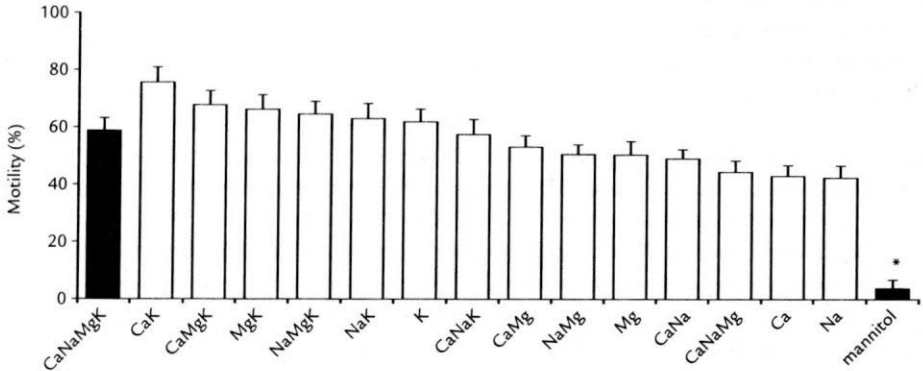


Figure 1. Effect of ions on sperm motility. Positive control: gray column; CaNaMgK; negative control: mannitol (black column). No significant differences compared to positive control  $p > 0.05$ ; \*significant differences compared to negative control,  $p < 0.05$ .

**EFFECT OF IONS ON PLASMA MEMBRANE POTENTIAL.** The fluorochrome Oxonol (Molecular Probes, Ore, USA) (bis-acid 1.3-dibutylbarbituric) trimethine oxonol (DiBAC4-3) that has a high affinity for membrane lipids at rest, was used to detect changes in membrane potential. These changes induce the detachment of the fluorochrome and the consequent fading of the fluorescence. Non-motile spermatozoa ( $5 \times 10^6$  spermatozoa/ml) were incubated with  $1 \mu\text{M}$  oxonol for one hour at  $20^\circ\text{C}$ . The mean fluorescence intensity (MFI) was measured at 15 s intervals for 2 min in a flow cytometer FACS SORT (Becton Dickinson, USA) at 488 nm between 200 and 400 mW.

**EFFECT OF CHANNEL BLOCKERS ON MOTILITY AND ACTIVATION TIME.** To determine whether activation is due to the opening of ionic channels, competition blockers (Biomedicals, Germany) were used at different concentrations. Amiloride ( $100 \mu\text{M}$ ,  $500 \mu\text{M}$  and  $1 \text{mM}$ ) served as  $\text{Na}^+$  channel blocker; to block  $\text{K}^+$  channels 4-aminopyridine ( $5 \text{mM}$ ,  $25 \text{mM}$  and  $50 \text{mM}$ ) was used; Verapamil ( $100 \mu\text{M}$ ,  $500 \mu\text{M}$  and  $1 \text{mM}$ ) was used to block  $\text{Ca}^{2+}$  channels. The blockers were evaluated individually at each concentration and in combination at the highest concentrations. Semen samples were diluted 1:1000 in an isosmotic solution and incubated at  $20^\circ\text{C}$  for one hour, to allow ion interchange; then, the spermatozoa were activated with a solution containing the blockers. The gross motility and activation time were recorded. As negative control an activator solution without blockers was used.

**EFFECT OF  $\text{K}^+$  CONCENTRATIONS ON SPERM MOTILITY AND ACTIVATION TIME.** Nine isosmotic  $\text{K}^+$  solutions were tested to determine the optimal conditions for sperm activation: 1, 5, 10, 15, 35, 70, 105 and  $140 \text{mM}$ . Additionally, two other control solutions were included: one without  $\text{K}^+$  but isosmolar to seminal plasma and the other with  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$  and  $\text{Na}^+$  at the same concentrations as in the seminal plasma.

**STATISTICAL ANALYSIS.** A General Linear Model was used to study the quantitative stochastic relationship among the dependent (gross motility (%), activation time (s), average intensity of fluorescence (AIF) and the independent variables (ionic balance, channel blockers,  $K^+$  concentrations) using the Tukey and Kramer test ( $p < 0.05$ ). The variables, motility and AIF were transformed to  $\log_{10}$  and  $\log(100+X)$ , respectively (Sas Institute, I., Cary, Nc 2002).

## RESULTS

### EFFECT OF IONS ON GROSS MOTILITY AND ACTIVATION TIME.

All the ionic solutions tested induced motility oscillating between 42 and 75% while in the negative control the motility was only 4% ( $p < 0.005$ ). The highest motility (57-75%) was obtained with solutions containing  $K^+$ , but there was no difference with the positive control (58%) (Fig. 1). The activation time varied from 56 to 94 s and the difference was significant ( $p < 0.05$ ) compared to the negative control (16 s). The solutions yielding the longest activation time were:  $Na^+Mg^{2+}Ca^{2+}$  (94 s) and  $Na^+Mg^{2+}$  (79 s) but no significant difference was found ( $p > 0.05$ ) with the positive control (78 s; Fig. 2).

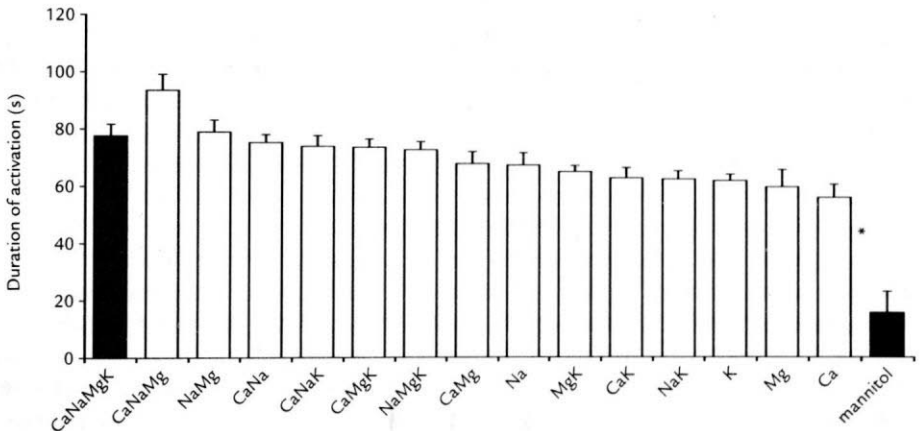


Figure 2. Effect of ions on the duration of motility. Positive control: gray column; CaNaMgK; negative control: mannitol (black column). No significant differences compared to positive control  $p > 0.05$ ; \*significant differences compared to negative control,  $p < 0.05$ .

### EFFECT OF IONS ON PLASMA MEMBRANE POTENTIAL.

All of the solutions, including controls induced the same change in membrane potential ( $p > 0.5$ ). This change was noticeable starting 15 s after activation, but the peak of sperms undergoing a change occurred at 30 s and remained steady up to 120 s ( $p < 0.05$ ; Fig. 3).

### EFFECT OF CHANNEL BLOCKERS ON MOTILITY.

Blockers of  $K^+$ ,  $Ca^{2+}$  and  $Na^+$  channels, either individually or in combination did not affect motility but reduced the duration of activation, independently of the ionic concentration ( $p > 0.05$ ; Fig. 4).  $Ca^{2+}$  and  $K^+$  channel blockers shortened the duration of

activation from 63 (control) to 18 s, when the highest blocker concentration was used (10 times concentrated;  $p < 0.05$ ; Fig. 4). Blockers of  $\text{Na}^+$  channels showed a low effect, from 63 to 44 s ( $p < 0.05$ ; Fig. 5).

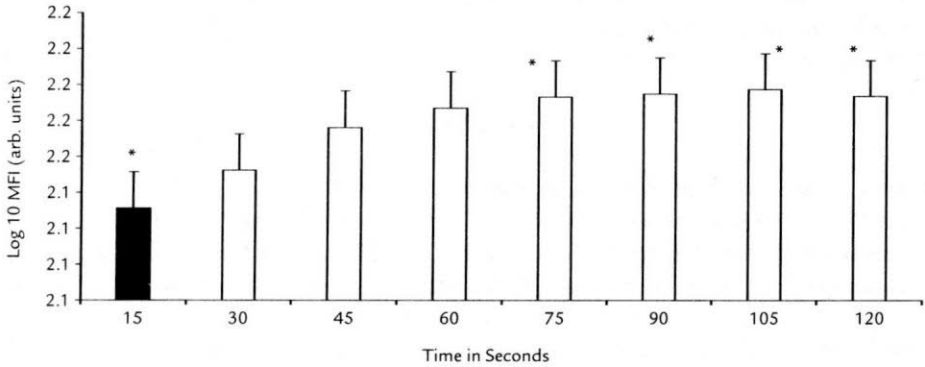


Figure 3. Variation in the membrane potential with time, after activation of sperm sample. \*Significant differences compared to 15 s,  $p < 0.05$ .

#### EFFECT OF $\text{K}^+$ CONCENTRATION ON MOTILITY AND DURATION OF MOTILITY.

$\text{K}^+$  between 1 and 70 mM had no effect on motility but there was a reduction from 73-83% (positive control) to 23-54% when the concentration was increased to 105 mM and to 0-11% when it was increased to 140 mM ( $p < 0.05$ ; Fig. 6). However, the duration of activation did not change significantly at different  $\text{K}^+$  concentrations (Fig. 7).

### DISCUSSION

When an osmotic shock is produced to activate spermatozoa, there is a reduction of extracellular  $\text{K}^+$  probably inducing the mobilization of the ion from mitochondria through  $\text{K}^+/\text{H}^+$  interchangers (Garlid, 1994) to the external milieu. The increase in positive charges induced by the efflux of  $\text{K}^+$  generates a change of membrane potential (Ohta and Shinriki, 1998) and this leads to an increase in cytosolic  $\text{Ca}^{2+}$  entering through voltage channels (Krasznai *et al.*, 2000) and also possibly to the liberation of the  $\text{Ca}^{2+}$  reserves from mitochondria through the voltage or ligand channels (Carafoli, 2003). The increase of intracellular  $\text{Ca}^{2+}$  would thereby dissipate the signaling route by initiating motility.

It was found that extracellular  $\text{K}^+$  and  $\text{Ca}^{2+}$  are important for the initiation of motility (solutions with  $\text{K}^+$  and  $\text{Ca}^{2+}$ , compared to the negative control, Fig. 1;  $p < 0.05$ ). On the other hand,  $\text{Na}^+$  and  $\text{Mg}^{2+}$  maintain this motility, but not by voltage-dependent channels since blocking these ions did not affect the initiation of motility. The change in membrane potential is due in part to the low extracellular osmolality. However, this factor alone is not sufficient because in the absence of extracellular ions (negative control) the potential changed but not the initiation of motility. Therefore, initiation of motility in *B. henni* could be regulated by the ion flux through channels independently of changes in the membrane potential since it occurs with stretching, interchanging or ligand channels.

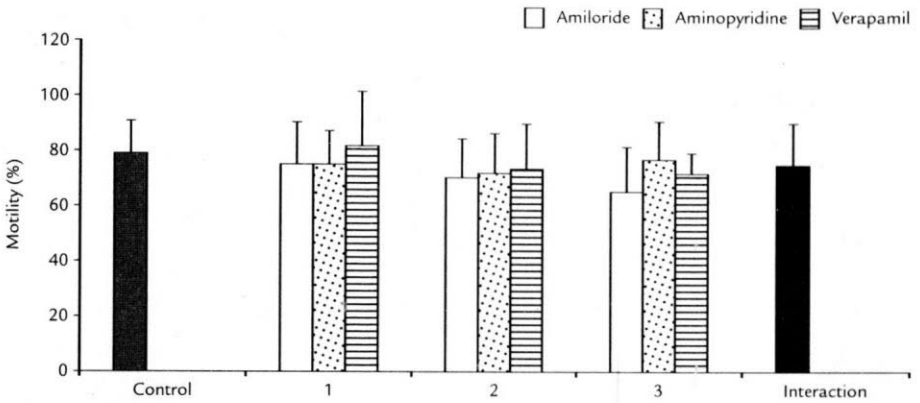


Figure 4. Effect of three different concentrations of channel blockers on sperm motility. Amiloride: Na channel blocker; 4-Aminopyridine: K channel blocker; Verapamil: Ca influx inhibitor. 1: Amiloride 100  $\mu$ M, Aminopyridine 5 mM and Verapamil 100  $\mu$ M; 2: Amiloride 500  $\mu$ M, Aminopyridine 25 mM and Verapamil 500  $\mu$ M; 3: Amiloride 1 mM, Aminopyridine 50 mM and Verapamil 1 mM. Interaction: combination of 3 blockers, Amiloride 1 mM, Aminopyridine 50 mM and Verapamil 1 mM. Control: without blockers (no significant differences compared to control,  $p > 0.05$ ).

This study illustrates the importance of extracellular  $K^+$  for sperm motility in *B. henni* as opposed to results with other fresh water fishes such as *Onchorhynchus mykiss* (Morisawa *et al.*, 1983) and *Clarias gariepinus* (Mansour *et al.*, 2002) in which  $K^+$  inhibits motility, or in *Morone saxatilis* (He *et al.*, 2004) where this ion has no effect. When only  $K^+$  was tested, even up to twice the concentration found in normal seminal plasma, initiation of motility was observed, but at higher concentrations motility did not begin and this observation deserves further experimentation and analysis. As opposed to previous reports on salmonids (Billard and Coson, 1992) and the common carp *Cyprinus carpio* (Krasznai *et al.*, 2000) demonstrating that extracellular  $Ca^{2+}$  is necessary to initiate motility, our results in *Brycon* indicate that  $Ca^{2+}$  is not necessary, and are in agreement with those of others (He *et al.*, 2004) studying *Morone saxatilis*.  $Ca^+$  favors motility synergistically with  $K^+$  (Fig. 1), as proposed previously for *C. carpio* (Krasznai *et al.*, 2000); the authors concluded that when  $K^+$  leaves the cells the consequent change in potential activates  $Ca^{2+}$  voltage-dependent channels, as opposed to what occurs in *B. henni*, where the participation of other types of  $Ca^{2+}$  channels is suggested. The ionic solutions generating the highest percentages of motility are the same as those that generate the maximum number of sperms with a change of potential in their membranes (Fig. 1; Fig. 3). Interestingly, these solutions are not the same as those that induce the longest activation times (Fig. 2), suggesting that these two events use different mechanisms.

The longest motility times were observed with solutions in which  $Na^+$  and  $Mg^{2+}$  were combined (Fig. 2), in agreement with the report of others (Mansour *et al.*, 2002) in *Clarias gariepinus* who suggest that the intracellular pH oscillates with the activation time and conclude that the antiporter  $Na^+/H^+$  is involved in pH regulation depending on osmolality and extracellular ions. Without extracellular  $Na^+$  the antiporter  $Na^+/H^+$  cannot activate itself reducing the activation time.  $Mg^{2+}$  maintains motility for longer periods per-



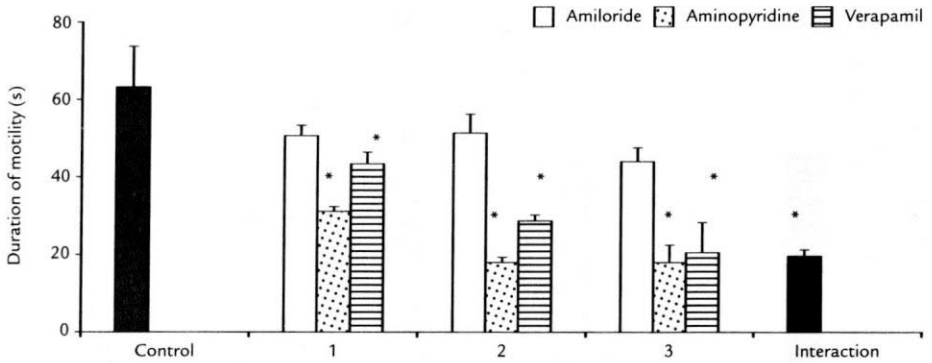


Figure 5. Effect of three different concentrations of channel blockers on duration of motility. Amiloride: Na channel blocker; 4-Aminopyridine: K channel blocker; Verapamil: Ca channel blocker. 1: Amiloride 100  $\mu$ M, Aminopyridine 5 mM and Verapamil 100  $\mu$ M; 2: Amiloride 500  $\mu$ M, Aminopyridine 25 mM and Verapamil 500  $\mu$ M; 3: Amiloride 1 mM, Aminopyridine 50 mM and Verapamil 1 mM. Interaction: combination of 3 blockers, Amiloride 1 mM, Aminopyridine 50 mM and Verapamil 1 mM. Control: without blockers. (\*Significant differences compared to control  $p < 0.05$ ).

haps through the activation of adenylyl cyclase to produce cAMP, that participates in the activation of protein movement through ATP hydrolysis with  $Mg^{2+}$  as a cofactor (Inaba *et al.*, 1998; Barbiroli *et al.*, 1999; Cosson *et al.*, 1999). This mechanism is not completely understood but it is thought that  $Mg^{2+}$  modulates the opening and closing of mitochondrial channels thus creating the conditions for flagellar movement (Shi *et al.*, 2002).

The duration of motility in *B. henni* (Fig. 5) may be associated to ionic regulation through different ion channels, contrary to what occurs in ciprinids that use a single type of channel (Krasznai *et al.*, 2003). This parameter decreases significantly in *B. henni* whatever blocker is used suggesting that 1) the intervening channels are important for motility although other channels may be involved that are efficient in initiating rather than maintaining motility, and 2) the blockers require some time to saturate the channels before exerting their effect, such that motility is initiated but does

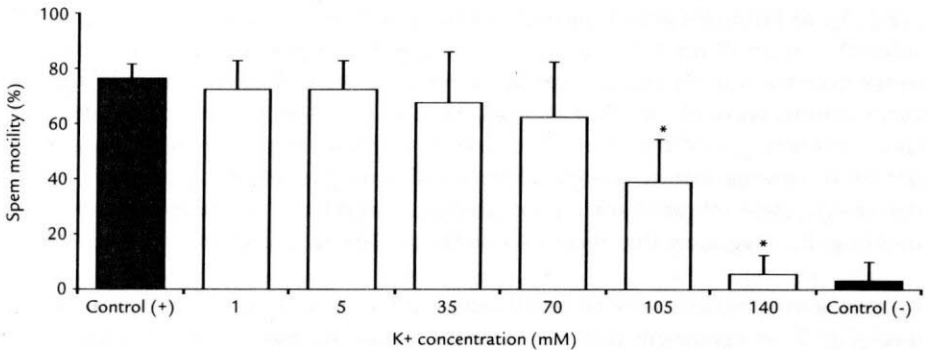


Figure 6. Effect of different concentrations of  $K^+$  (mM) on the acquisition of sperm motility. (\*Indicate significant differences compared to positive controls,  $p < 0.05$ ). Positive control: ion concentrations as found in seminal plasma (KCl: 37.3 mM, NaCl: 122.8 mM,  $CaCl_2$ : 0.87 mM,  $MgCl_2$ : 0.83 mM). Negative control: mannitol adjusted isosmolar solutions without ions.



In conclusion, activation of sperm motility in *Brycon henni* depends on a set of factors: 1) the ionic balance, with  $K^+$  playing a major role on the percentage of motile cells, and  $Na^+$  and  $Mg^{2+}$  on the duration of activation, and 2) the dynamics of the plasma membrane, indicated by the change in membrane potential, independently of the extracellular ionic balance.

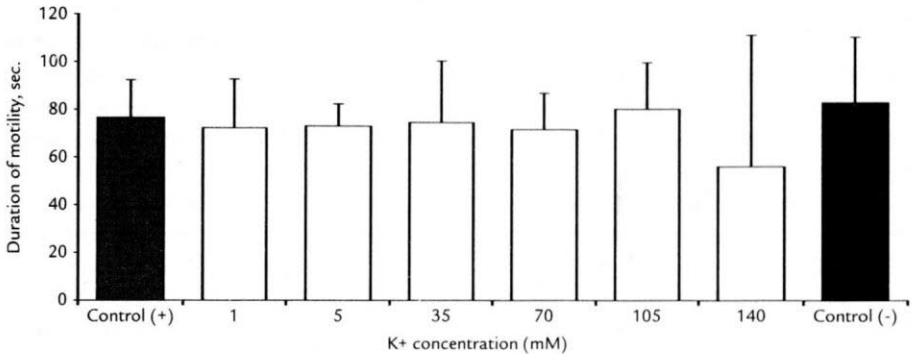


Figure 7. Effect of different  $K^+$  concentrations on the duration of sperm motility ( $p > 0.05$ ). Positive control: ion concentration as found in seminal plasma ( $KCl$ : 37.3 mM,  $NaCl$ : 122.8 mM,  $CaCl_2$ : 0.87 mM,  $MgCl_2$ : 0.83 mM). Negative control: mannitol adjusted isosmolar solutions without ions.

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