Pilot study methods

Two replicates of each cryosolution (supplementary table 1) were prepared in 2 ml cryogenic vials. The vials were first loaded with the diluents and milt dilution, after that the cryoprotectant was added. All milt samples were stripped and obtained from one male (2012). Fresh milt was used as a control.

Two procedures were tested in order to obtain an optimal freezing and thawing method. The freezing process was carried out in a styrofoam box (inside dimensions H x L x W= 21cm x 35.5cm x 23cm) with liquid nitrogen (-196º C) filled up to about 10 cm. During the freezing procedures, the cryogenic vials were placed upright on styrofoam trays at different heights using a metal wire attached to each vial and the tray. The tray and vials were placed on the liquid nitrogen in the styrofoam container in nitrogen gas for 10 minutes. They were then plunged directly into the liquid as the tray was turned over with a 20 cm long forceps. The vials were suspended in this position for at least 15 minutes. In the first freezing procedure, the height of the styrofoam tray was 2.5 cm, and, in the second freezing procedure, the height was changed to 4.5 cm. Subsequently the vials were thawed in water baths. The thawing temperature in the first procedure was 50˚ C and the duration time 1 minute. In the second procedure, the thawing temperature was reduced to 37oC and the duration time increased to 1.5 minute.

The motility score of milt samples was measured after the thawing procedure. We divided the movement of spermatozoa into five stages and classified each stage according to an arbitrary scale commonly used from 0 to 4 based on the percentage of moving cells (Viveiros, Jatzkowski & Komen, 2003; Rurangwa et al., 2004; Kainin et al., 2012) (Supplementary table 2).

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