Selection criteria of zebrafish male donors for sperm cryopreservation

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Summary

Selection criteria for sperm cryopreservation is highly relevant in zebrafish since sperm quality is particularly variable in this species. Successful cryopreservation depends on high quality sperm, which can only be ensured by the selection of breeders. Consequently, male selection and management are a priority to improve cryopreservation, and therefore, this study aimed to characterize optimal age and sperm collection frequency in zebrafish. For this purpose, males from wild type (AB) and from a transgenic line (Tg(*runx2:eGFP*)) were sampled at 6, 8, 12 and 14 months. For each age, sperm were collected at time 0 followed by samplings at 2, 7 and 14 days of rest. Sperm quality was assessed according to motility and membrane viability parameters. Quality assessment showed that *Tg*(*runx2:eGFP*) displayed

significantly higher motility than AB and younger males showed higher motility in both lines. Sperm collection frequency affected membrane viability. While AB fish recovered sperm viability after 14 days of rest, *Tg(runx2:eGFP)* could not recover. Consequently, it may be important to study the sperm quality of each zebrafish line prior to sperm cryopreservation. Taking in consideration the results achieved in both lines, sperm collection should be performed between 6 to 8 months of age with a minimum collection interval of 14 days.

Keywords: Sperm quality, donor age, sperm collection frequency, zebrafish sperm

Introduction

Successful cryopreservation depends on several factors, being the selection of high quality sperm one of the most important, since the freezing process induces damage that decrease sperm quality significantly.[1] Since high quality sperm is related to high quality breeders,[1]the selection of male donors is essential for broodstock management and cryopreservation programs. Zebrafish is an established model species maintained in laboratories worldwide and extensively used in numerous research fields, including biological and biomedical research.[2,3] As a consequence, in the past years, abundant and valuable wild type, mutant and transgenic zebrafish lines were established, posing problems in terms of space and management. To solve this issue, sperm cryopreservation can be used to support zebrafish facility management and to safeguard all those valuable genetic resources.[4] Zebrafish sperm cryopreservation was achieved for the first time more than 30 years ago by Harvey et al.[5]. However, until today, the most relevant issue for successful and reproducible results using cryopreservation is the lack of methodological standardization among

laboratories and rearing facilities, which translates into high variability in post-thaw sperm quality and *in vitro* fertilization success.

Sperm quality is defined by the ability of sperm to successfully fertilize an egg,[6,7] which is dependent on factors such as heritage,[8] spermiation period, favorable environmental conditions for activation of sperm motility,[9] parental age[10,11] and sperm output frequency.

It has been reported that the age of males affects both sperm production and quality,[10,12] resulting in lower reproductive success. This phenomenon is associated to the accumulation of *de novo* mutations in germ cells,[8,13] thus decreasing the genetic quality of gametes[9,13] and altering sperm functionality. It has been reported that in humans, age is associated to lower sperm volume, motility and percentage of normal sperm cells.[11,12] Furthermore, advanced parental age in several species is associated to a decline in sperm competition.[9,14] However, the decrease of sperm competition with age was not observed in teleosts, such as reported for sockeye salmon (*Oncorhynchus nerka*)[15] in *in vitro* fertilization experiments and for guppy (*Poecilia reticulata*) natural spawns.[16] Consequently, the effect of age on sperm quality is not similar in all vertebrates and should be investigated thoroughly in zebrafish to ensure the highest sperm quality for cryopreservation purposes.

From an animal welfare and practical point of view, the most convenient technique for sperm collection in a zebrafish facility is through abdominal massage, since it is a non-lethal technique.[17] In this way, sperm collection can be performed repeatedly on the same male.[18]

The influence of sperm collection frequency on sperm quality has been assessed in teleost species such as trout (*Salmo trutta*)[19,20] turbot (*Scophthalmus maximus*),[21] European sea bass (*Dicentrarchus labrax*),[22] and white fish (*Coregonus peled*).[23]

However, it is commonly accepted that an inappropriate sperm collection frequency affects sperm quality[7] and this must be determined for each species. Consequently, the assessment of an appropriate sampling frequency that allows a full recovery of sperm quality in zebrafish is essential for assisted reproduction purposes.

Motility is the most widely studied quality parameter in fish sperm[24,25] and although other analysis are needed to guarantee the status of spermatozoa, it is a useful tool to infer the probability of successful fertilization and to assess the previous mentioned factors.[25,26] Still, there are no universal sperm quality biomarkers, therefore, besides motility, other parameters are needed for an accurate quality analysis.[6,27] The viability of the plasma membrane is an important feature in spermatozoa since it characterizes the integrity of the cell.[24] Membrane alterations in spermatozoa can affect motility initiation (motility is triggered by membrane signaling), motility maintenance (loss of intracellular ATP) and the ability of the sperm nucleus to produce the first embryonic cell after fertilization.[26,28,29] In this way, analysis of sperm quality is a useful tool to select the most appropriate conditions to collect zebrafish sperm for cryopreservation and assisted reproduction purposes.

This study aimed at characterizing the optimal age for sperm collection in zebrafish and to evaluate the effect of the frequency of non-invasive sperm sampling on motility and plasma membrane viability.

Materials and Methods

Zebrafish maintenance

Zebrafish AB (wild type) and *Tg(runx2:eGFP)*,[30] with an AB background, were housed in a standard aquatic recirculation system (Zebtec®, Tecniplast, Italy) with 980 L of water, containing a biological filter (ceramic beads), mechanic filtration (50

μm), granular activated carbon filter and UV sterilization (180 000 μWs/cm^2) to maintain water quality. The water temperature (28 ± 0.5 ºC), conductivity (750 ± 70 μS) and pH (7.5 ± 0.2) were constantly monitored through automatic probes and water was partially replaced daily (10%). The fish room had a controlled photoperiod with a 14:10 h light:dark cycle, an independent air conditioning system (26 ± 1 °C) and an air extraction system to guarantee the air renewal in the room, maintaining the humidity close to 60%. Males and females were maintained separately in 3.5 L tanks. The fish were fed twice a day *ad libitum* with ZEBRAFEED® (Sparos Lda, Portugal) and *Artemia* nauplii (AF 480; INVE, Belgium). Unconsumed food and fish debris were removed daily.

All animal manipulations were performed in compliance with the Guidelines of the European Union Council (86/609/EU), according to the directive´s for protection of animals used for scientific research (2010/63/EU) and transposed to the Portuguese law for the use of laboratory animals on research (Decreto Lei n° 129/92 de 06 de Julho, Portaria n° 1005/92 de 23 de Outubro). All animal protocols and fish sampling procedures were performed by licensed researchers (Decreto Lei n°113/2013 de 7 de Agosto).

Sperm collection and quality analysis

On the day prior to sperm collection, males and females were placed in 1 L breeding tanks in 1:1 sex-ratio (Tecniplast, Italy) and maintained separated while sharing the same water, in order to promote hormonal stimulation for improved release of gametes. Males were anesthetized with 0.168 mg/mL of tricaine methane-sulfonate solution (MS-222) (Sigma Aldrich, Spain) prepared according to Westerfield[31] and sperm was collected by abdominal massage using a glass capillary tube connected to

a mouth piece. Sperm was immediately diluted with 10 μL of sterilized and filtered (0.20 μm) Hank's Balanced salt solution (HBSS) at 300 mOsm/Kg,[32] to prevent motility activation, in accordance with previous studies.[33] After sperm collection, the samples were maintained at 4 ºC in the dark until quality analysis was performed, while the males were recovered from the anesthesia in a clean system water and returned to the rearing tanks. Sperm motility was evaluated using Computer Assisted Sperm Analysis (CASA) (Proiser, Spain). To evaluate motility parameters, 0.5 μL of sperm at room temperature was placed on a Makler chamber under a 10 x negative phase-contrast objective (Nikon E200, Tokyo, Japan) and immediately activated with 5 μL of filtered and sterilized system water at 28 ± 1 ºC. Motility was recorded each 10 s post activation, during 1 min for each sample. The images were captured with a Basler camera A312f (Basler Afc, Germany). Total motility (TM, %); progressive motility (PM, %), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s) and linearity (LIN, %) were determined to assess sperm quality. Only those spermatozoa with VCL > 10 μm/s were considered motile.

To evaluate spermatozoa membrane viability, the percentage of viable cells was quantified using the fluorescent dyes propidium iodide (PI) (Invitrogen, Spain) and SYBR 14 (Invitrogen, Spain). Before the addition of the fluorescent dyes, the sperm sample was re-diluted (1:10) in HBSS, to reduce cell concentration. Incubation with 5 μM SYBR 14 and 220 μM PI were performed in the dark at 4 ºC for 5 min. Cell viability was quantified under an epifluorescence microscope (Nikon E200, Tokyo, Japan), equipped with triple excitation filter block DAPI-FITC-Texas Red (excitation filter wavelengths: 395–410 nm (bandpass, 403 CWL), 490–505 nm (bandpass, 498 CWL), and 560–580 nm (bandpass, 570 CWL)). Dead cells with disrupted membrane labelled in red (PI stained cells) and live cells labelled in green

(SYBR 14 stained cells) were counted, and the percentage of viable cells was determined. At least 100 cells per slide were counted, and two slides per sample and per condition were observed.

Effect of age and sperm collection frequency on sperm quality

Males from wild-type (AB) and *Tg(runx2:eGFP)* zebrafish lines were sampled for sperm collection. To study the effect age on AB (N = 90) and *Tg(runx2:eGFP)* (N = 85) lines on sperm motility, males were sampled with 6 (N = 49), 8 (N = 30), 12 (N = 45) and 14 (N = 51) months. The viability of the membrane of the same males was analyzed at 6 (N = 43), 8 (N = 25), 12 (N = 45) and 14 (N = 48) months of age.

To evaluate the effect of sperm collection frequency on sperm motility of AB (N = 90) and *Tg(runx2:eGFP)* (N = 85) zebrafish lines, for each line and each age, males were sampled for the first time as baseline (0) (N = 51) and sperm collections were performed after 2 (N = 37), 7 (N = 55) and 14 (N = 32) days of rest. For the same males, the viability of the membrane was analyzed when males were sampled for the first time (0) (N = 45), and sperm collections performed after 2 (N = 31), 7 (N = 51) and 14 (N = 34) days of rest.

Sperm motility parameters of the individual males was assessed through CASA system each 10 s post-activation during 1 min, for determining TM, PM, VCL, VSL and LIN. Viability of the plasma membrane was evaluated as previously described.

Data analysis

Due to the high number of variables related to sperm motility measured for each sample (5 motility parameters × 6 post-activation times = 30 motility-related variables), we started by evaluating their degree of redundancy, using Principal

Component Analysis (PCA), in order to assess whether it was possible to aggregate all these 30 variables into a small number of variables, without significant information loss. After a preliminary exploratory analysis, we observed that the LIN variables displayed very low variance, except for sperm samples with extremely low motility and no particularly relevant linear correlation with the other variables. As such, we have no longer considered the LIN parameters for analysis. In contrast, all other parameters (TM, PM, VCL and VSL) displayed a high degree of positive correlation among them, which was reflected by the fact that it is possible to aggregate these 24 variables into a single variable (PC1), that still retains 52% of observed variation (after mean-centering and auto-scaling of these variables, to ensure that PCA does not give preference to higher variance variables) and which can be interpreted as a general "motility index".

This "motility index" (i.e. the first component of the PCA analysis) consisted of a weighted mean of these motility measurements (after standardization), which was used for further ANOVA analysis.

SPSS 18.0 software was used for statistical analysis. Data were expressed as means ± 95% C.I. (95% of confidence interval of the mean), and normalized by arcsine transformation when results were expressed as percentages. Statistical differences between treatments were detected by ANOVA and Student-Newman-Keuls (SNK) multiple comparison post-hoc tests ($p < 0.05$). A three-way ANOVA (SNK, $p < 0.05$) was performed on all motility (PC1) data. For each zebrafish line, a two-way ANOVA (SNK, $p < 0.05$) was applied to evaluate the effect of age and sperm collection frequency on sperm quality.

Results

A three-way ANOVA (SNK, $p < 0.05$) was performed on motility (PC1) data, which showed that the zebrafish line was the factor with largest main effect on motility (Table 1), since *Tg(runx2:eGFP)* had significantly higher sperm motility when compared to AB (Figures 1a and 1b, 2a and 2b). Given the high number of significant interaction effects observed between factors (line, age and stripping frequency) (Table 1), which impair interpretation of the main treatment effects, a two-way ANOVA (SNK, $p < 0.05$) was applied for each line independently, to study the effects of age and stripping frequency (along with possible interactions between these factors). Both lines showed a consistent main effect of age, where younger males (6 and 8 months) had significantly higher sperm motility when compared to older males (12 and 14 months) (Figure 1a and 1b). The results of stripping frequency on sperm motility were also consistent in both lines, with no significant effect being observed (Table 1; Figure 2a and b). In both lines, there was an age interaction with frequency effect, which means that stripping frequency had an effect on sperm motility that depends on age (Figure 3a and b).

The percentage of viable cells was analyzed with a three-way ANOVA (SNK $p < 0.05$) after arcsin transformation, with the factor zebrafish line displaying no effect on sperm viability (Table 2). However, the high number of interaction effects impaired a clear interpretation of the effect of age and stripping frequency on sperm viability, so, as previously, a two-way ANOVA (SNK, $p < 0.05$) was applied to each line independently to study the effect of age and stripping frequency. Sperm viability in the AB line showed no significant differences between all the studied ages (Figure 4a and b), while in the *Tg(runx2:eGFP)* line a significantly higher sperm viability was observed at 8 months of age. In the AB line, males sampled for the first time had significantly higher sperm viability when compared to samples collected 2

and 7 days after first stripping, but it was not significantly different from 14 days after stripping (Figure 5a and b). Consequently, AB males were able to recover membrane viability 14 days after stripping. On the other hand, *Tg(runx2:eGFP)* males were not able to recover membrane viability 14 days after stripping, since males in the first sampling point had significantly higher sperm viability compared with males after 7 and 14 days of recovery (Figure 5b).

Discussion

The selection of sperm donors is highly relevant for cryopreservation, since it can help reducing post-thaw variability in zebrafish sperm used for *in vitro* fertilization. In captivity, zebrafish has a natural longevity of 42 to 66 months, depending on reproductive effort and caloric intake.[34] In our study, younger zebrafish males (6 to 8 months) showed significantly higher sperm motility when compared to older males (12-14 months). In agreement, Johnson et al.[35] observed that older zebrafish males had a decline in sperm production and motility, although displaying higher offspring survival. In guppy, male age affected negatively sperm morphology, velocity and sperm number but not membrane viability. Older males had slower sperm, with longer flagellum and higher sperm volume than younger males,[10] but these differences did not affect sperm competition success when compared to younger males. It is interesting to observe that both in zebrafish and in guppy there is a decrease in sperm motility in older males, which might be associated to the accumulation of oxidized proteins and a decline in mitochondrial functionality.[36] Mitochondria aging related alterations are among the most remarkable features observed in senescent cells. Mitochondrial oxidation is the major source of oxidation lesions accumulated with age, affecting mitochondrial energy metabolism, which is

essential for male reproductive function. The damage produced by excessive reactive oxygen species (ROS) in the sperm membrane cause reduced sperm motility and impairs its ability to fuse with the oocyte.[37] Oxidative stress reduces sperm motility and viability[4,38–40] and could be one of the possible explanations for the lower motility in older zebrafish males.

Zebrafish are hierarchical fish with dominant-subordinate relationships, which are related with body size and levels of aggression, associated to reproductive success.[41] Two distinct reproductive strategies have been identifies in males: territorial or actively pursuing the female, having the first strategy higher success in breeder populations at low densities.[42] The hierarchical relationships established among zebrafish are strongly connected to sperm competition, which is a post-copulatory selection that occurs when females breed with multiple males in the same reproductive episode. In this process, sperm from rival males compete to fertilize the oocytes.[43] In sperm competition for fertilization, there is a strong selection for spermatozoa quality parameters that enhance fertilization success, such as sperm quantity and quality.[44,45] Therefore, the reproductive set-up established to determine sperm quality is extremely important since changes in the social environment affect rapidly sperm competition and therefore sperm quality. Zajitschek et al.,[46] found that under high sperm competition environment (two males and one female), males display higher sperm motility and velocity than reproductive set-ups where one male was available to two females (low sperm competition environment). In our study, males of each age where permanently maintained separated from females and the reproductive set-up was established in breeding tanks in a sex ratio of 4:4 to stimulate reproduction and collect the sperm on the following day.

Therefore, the results obtained with our experimental design emphasize the effect of age and sperm collection frequency in sperm quality reducing biases associated to sperm competition effects.

Considering this information, it largely explains the differences observed between studies on the effects of zebrafish age on reproduction and sperm quality. Not only are the samples highly heterogeneous, but they also manifest adaptations and different investments in gamete production according to the social environment and hierarchical relationships. A characterization of the effect of zebrafish age on sperm competition should be undertaken in the future.

Spermiogenesis is a complex and highly regulated process, where diploid cells called spermatogonia proliferate and differentiate onto mature spermatozoa through mitosis, meiosis and spermiogenesis.[47] Zebrafish spermatogenesis has a cystic pattern with one of the teleosts fastest spermiogenesis cycle taking only 6 days to reach spermatozoa full maturation.[47] Reinardy et al.[48] observed that with stripping frequencies with a maximum of 7 days of rest, the DNA integrity was not altered, despite the fact that sperm concentration was affected. Consequently, it was necessary to determine the adequate stripping frequency that allows the full recovery of sperm quality. Our data showed that stripping frequency does not affect sperm motility, though it does affect membrane viability. The AB zebrafish line was able to recover the initial membrane viability after 14 days of rest. However, this recovery was not observed in the transgenic line, where sperm membrane viability was still decreased after 7 and 14 days of rest.

In our study recovery time in younger fish is seemingly faster than in older fish, which is observed through the interaction effects of age and stripping frequency: older fish display lower sperm motility when the recovery time between collections

is short, while sperm motility for younger fish seems insensitive to the recovery time between collections. Consequently, it is highly advisable to respect 14 days of rest between sperm collection events, particularly in older fish. The fact that younger fish are less susceptible to cellular distress, related to sperm collection events, reinforces the selection criteria of using younger fish for sperm collection.

The experimental design used in our work allowed a better comprehension of the interaction between treatments, that would not be possible otherwise. Both zebrafish lines are commonly used in zebrafish facilities and the transgenic model was used as comparison between wild type fish breeders with genetic modified zebrafish lines. The *Tg(runx2:eGFP)* line has an AB background and expresses the *Tg(runx2:eGFP)* transcription factor which is related to osteoblast differentiation but also to the regulation of cell proliferation. Although most studies on zebrafish sperm cryopreservation and assisted reproduction are performed with wild type lines, its application is most useful in transgenic and mutant lines, and their particularities are generally unknown or disregarded.

Throughout our experiments the *Tg(runx2:eGFP)* transgenic line had systematically higher sperm motility when compared to AB line. The fact that *Tg(runx2:eGFP)* fish displayed significantly higher sperm motility but lower capacity to recover membrane viability at 7 and 14 days after sperm collection, suggests the existence of relevant differences between zebrafish lines in terms of sperm quality and susceptibility to damage. Consequently, each zebrafish transgenic and mutant line should be investigated prior to the establishment of sperm cryopreservation programs.

The knowledge obtained by this work allows the determination of suitable zebrafish age and sperm collection frequencies to obtain the highest sperm quality possible to

facilitate cryopreservation procedures respecting the 3 R´s principle Therefore, we consider that males between 6 to 8 months of age have the highest sperm quality and at least 14 days of rest should be respected between sperm collection events.

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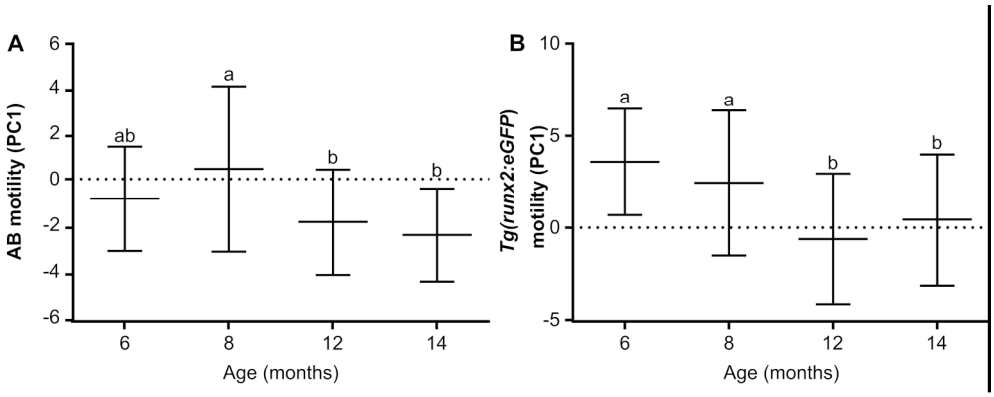

FIG 1 – Zebrafish sperm motility (PC1) according to age of: a) AB line at 6 (N = 26), 8 (N = 13), 12 (N = 27) and 14 (N = 24) months of age. b) Tg(runx2:eGFP) line at 6 (N = 23), 8 (N = 17), 12 (N = 18) and 14 (N = 27) months of age. The analysis was performed with the baseline data of the first sampling. Bars represent 95% of confidence interval and statistical differences (two-way ANOVA-SNK, $P < 0.05$) between fish age are represented with letters.

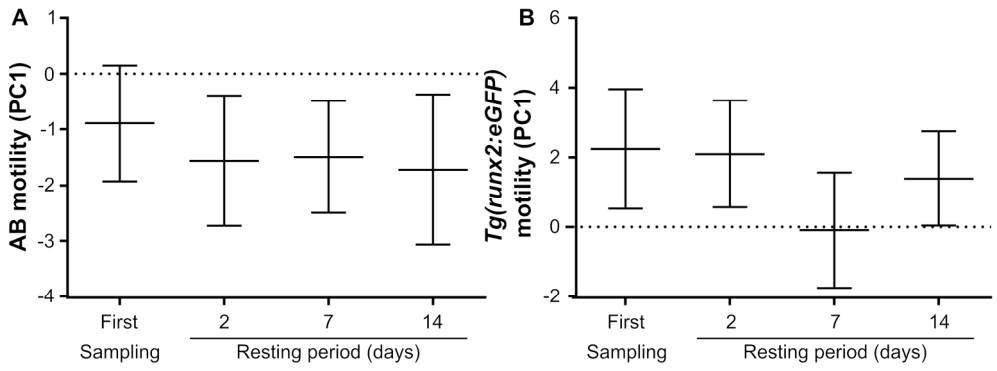

FIG 2 – Zebrafish sperm motility (PC1) after different sperm stripping frequencies. The number of the stripping frequency is related to the time of rest between sperm collections. After the first sampling sperm was collected after 2, 7 and 14 days of rest between samplings. The analysis was performed in: a) AB line at first stripping (N = 26) and after 2 (N = 13), 7 (N = 27) and 14 (N = 24) days of rest, b) Tg(runx2:eGFP) line at first sampling (N = 23) and after 2 (N = 17), 7 (N = 18) and 14 (N = 27) days of rest. The analysis was performed with males with 6 months of age. Bars represent 95% of confidence interval and statistical differences (two-way ANOVA-SNK, $P < 0.05$) between sperm collection frequencies are represented with letters.

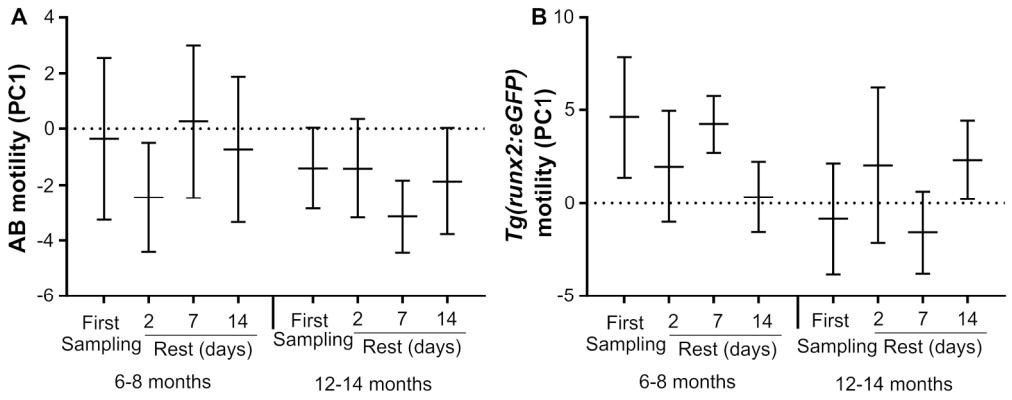

FIG 3 – Effect of the interaction in zebrafish sperm quality between males age and sperm stripping frequency in a) AB wild type line and b) Tg(runx2:eGFP) line. The number of the stripping frequency is related to the time of rest between sperm collections where. Analysis was performed in the first sampling (AB N = 24; Tg(runx2:eGFP) N = 27) and after 2 (AB N = 14; Tg(runx2:eGFP) N = 23), 7 (N = 33; Tg(runx2:eGFP) N = 22) and 14 (N = 19; Tg(runx2:eGFP) N = 13) days of rest between samplings. This analysis is related to males with 6 to 8 months (AB N = 39; Tg(runx2:eGFP) N = 40) and 12 to 14 months (AB N = 51; Tg(runx2:eGFP) N = 45) of age. Bars represent 95% of confidence interval.

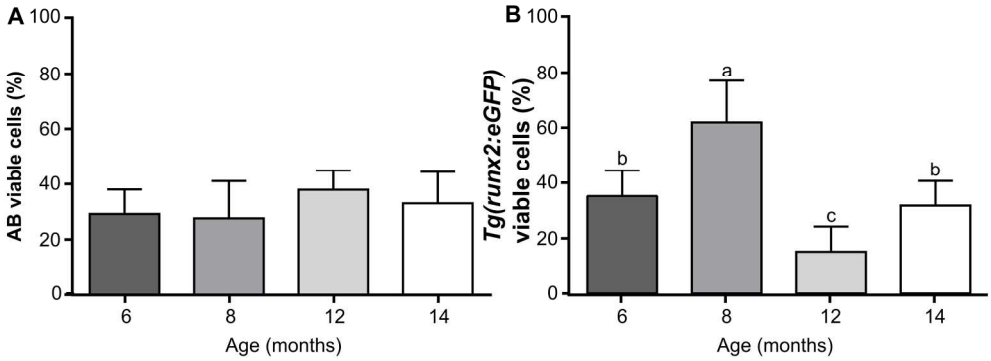

FIG 4 – Zebrafish sperm viability (%) according to age of: a) AB line at 6 (N = 20), 8 (N = 13), 12 (N = 27) and 14 (N = 21) months of age and b) sperm viability (%) of Tg(runx2:eGFP) line at 6 (N = 23), 8 (N = 12), 12 (N = 18) and 14 (N = 27) months of age. The analysis was performed with the baseline data of the first sampling. Bars represent 95% of confidence interval and statistical differences (two-way ANOVA-SNK, $P < 0.05$) between fish age are represented with letters.

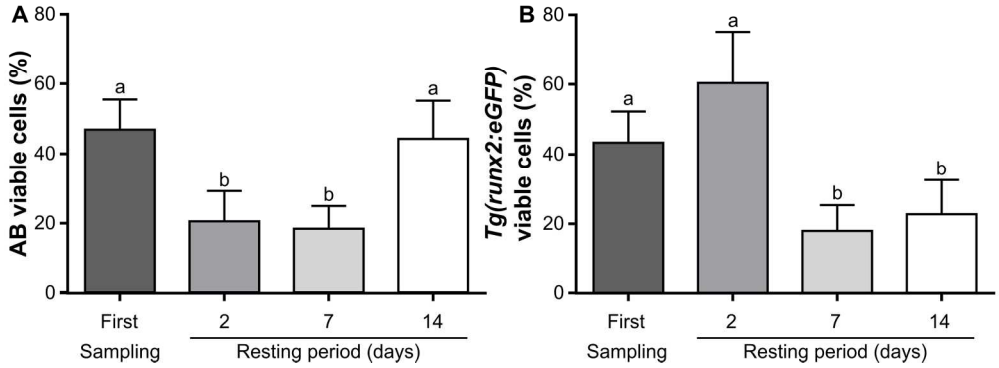

FIG 5 – Zebrafish sperm viability (%) after different sperm stripping frequencies. The number of the stripping frequency is related to the time of rest between sperm collections. After the first sampling sperm was collected after 2, 7 and 14 days of rest between samplings. The analysis was performed in: a) AB line at the first sampling (N = 23) and 2 (N = 18), 7 (N = 29) and 14 (N = 11) days of rest, b) Tg(runx2:eGFP) line at the first sampling (N = 22) and 2 (N = 13), 7 (N = 22) and 14 (N = 23) days of rest. The analysis was performed with males with 6 months of age. Bars represent 95% of confidence interval and statistical differences (two-way ANOVA-SNK, $P < 0.05$) between sperm collection frequencies are represented with letters.

FIG 1 – Zebrafish sperm motility (PC1) according to age of: a) AB line at 6 (N = 26), 8 (N = 13), 12 (N = 27) and 14 (N = 24) months of age. b) *Tg(runx2:eGFP)* line at 6 (N = 23), 8 (N = 17), 12 (N = 18) and 14 (N = 27) months of age. The analysis was performed with the baseline data of the first sampling. Bars represent 95% of confidence interval and statistical differences (two-way ANOVA-SNK, $P < 0.05$) between fish age are represented with letters.

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FIG 3 – Effect of the interaction in zebrafish sperm quality between males age and sperm stripping frequency in a) AB wild type line and b) *Tg(runx2:eGFP)* line. The number of the stripping frequency is related to the time of rest between sperm collections where. Analysis was performed in the first sampling (AB N = 24; *Tg(runx2:eGFP)* N = 27) and after 2 (AB N = 14; *Tg(runx2:eGFP)* N = 23), 7 (N = 33; *Tg(runx2:eGFP)* N = 22) and 14 (N = 19; *Tg(runx2:eGFP)* N = 13) days of rest between samplings. This analysis is related to males with 6 to 8 months (AB N = 39; *Tg(runx2:eGFP)* N = 40) and 12 to 14 months (AB N = 51; *Tg(runx2:eGFP)* N = 45) of age. Bars represent 95% of confidence interval.

FIG 4 – Zebrafish sperm viability (%) according to age of: a) AB line at 6 (N = 20), 8 (N = 13), 12 (N = 27) and 14 (N = 21) months of age and b) sperm viability (%) of *Tg(runx2:eGFP)* line at 6 (N = 23), 8 (N = 12), 12 (N = 18) and 14 (N = 27) months of age. The analysis was performed with the baseline data of the first sampling. Bars represent 95% of confidence interval and statistical differences (two-way ANOVA-SNK, $P < 0.05$) between fish age are represented with letters.

FIG 5 – Zebrafish sperm viability (%) after different sperm stripping frequencies. The number of the stripping frequency is related to the time of rest between sperm collections. After the first sampling sperm was collected after 2, 7 and 14 days of rest between samplings. The analysis was performed in: a) AB line at the first sampling (N = 23) and 2 (N = 18), 7 (N = 29) and 14 (N = 11) days of rest, b) *Tg(runx2:eGFP)* line at the first sampling (N = 22) and 2 (N = 13), 7 (N = 22) and 14 (N = 23) days of rest. The analysis was performed with males with 6 months of age. Bars represent 95% of confidence interval and statistical differences (two-way ANOVA-SNK, $P < 0.05$) between sperm collection frequencies are represented with letters.

TABLE 1– Zebrafish sperm motility analysis of Principal component (PC1) related to age, stripping frequencies and their interactions in AB and *Tg(runx2:eGFP)* line.

Three-way ANOVA (p value < 0.005)	Motility (PC1)
Line	<0.001*
Age	<0.001*
Frequency	0.437
Strain*age	0.480
Strain*frequency	0.212
Age*frequency	<0.001*
Strain*age*frequency	0.024*
AB (two-way ANOVA) (p value < 0.005)	
Age	0.025*
Frequency	0.825
Age*frequency	0.002*
runx2 (two-way ANOVA) (p value < 0.005)	
Age	0.001*
Frequency	0.179
Age*frequency	0.001*

Significant differences (three-way ANOVA (SNK, $p < 0.05$)) are represented with asterisk.

TABLE 2– Zebrafish sperm membrane viability analysis related to age, stripping frequencies and their interactions in AB and *Tg(runx2:eGFP)* line.

Three-way ANOVA (p value < 0.005)	Viability
Line	0.267
Age	0.007*
Frequency	<0.001*
Strain*age	0.083
Strain*frequency	<0.001*
Age*frequency	<0.001*
Strain*age*frequency	0.346

AB (two-way ANOVA) (p value < 0.005)	
Age	0.145
Frequency	<0.001*
Age*frequency	0.003*

runx2 (two-way ANOVA) (p value < 0.005)	
Age	<0.001*
Frequency	<0.001*
Age*frequency	0.001*

Significant differences (three-way ANOVA (SNK, $p < 0.05$)) are represented with asterisk.