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Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*).

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Abstract

The use of high quality gametes from both males and females during in vitro fertilization (IVF) trials is an essential step in order to achieve high fertilization and hatching rates. Although aquaculture hatcheries have focused more on egg rather than spermatozoa quality, some studies have demonstrated that sperm quantity and quality have a great influence both on fertilization/hatching success and the subsequent development of the embryo and larvae.

In this study we have demonstrated that sperm/egg ratio and sperm quality are factors strongly related to each other in the pufferfish (*Takifugu niphobles*). Our results suggest that both factors should be taken into account as unique interrelated elements, making possible to obtain high fertilization rates using a successful combination of small amount of high quality sperm or high amount of low quality sperm.

In addition, coefficients of correlation and determination among all the sperm motion parameters provided by a CASA system and fertilization/hatching rates were estimated for the first time in a marine species. Positive significant correlations were found in some parameters such as total and progressive motility (0.68 and 0.7 respectively). However, curvilinear velocity (VCL), straight line velocity (VSL) and average velocity (VAP) showed the highest coefficients of correlation (0.82, 0.8, and 0.81, respectively). In this respect, spermatozoa velocity appears to be a key factor in the fertilization process, especially when the number of spermatozoa per egg is limited in the aqueous environment.

Keywords

Fugu; Sperm; Motility; CASA; Velocity; In Vitro Fertilization

1. Introduction

The pufferfish (*Takifugu niphobles*) is a teleost fish with a wide distribution in the Northwest Pacific Ocean. This species is one of around 24 pufferfish species in the tetraodontine genus *Takifugu*, and it presents own interesting features to preserve it: *i*) it is placed on the IUCN Red List due to the fact that its current population is not well known, making it a possible endangered species (Roberts, 1996); and *ii*) another closed species, like *Takifugu rubripes*, is widely-kept by scientists as a model organism (Aparicio et al., 2002) so *Takifugu niphobles* could be used like this due to its small and similar genome (Brenner et al., 1993)

Breeding in captivity of *Takifugu spp.* involves the handling of captive fish broodstocks and the collection of gametes both from males and females for the application of *in vitro* fertilization (IVF). This technique is essential for the reproductive manipulation of some fish species such as *Takifugu niphobles*, which displays an exclusive and complex spawning behavior because spawning does not happen spontaneously under cultured conditions.

The general hatchery protocol for IVF in fish involves mixing quiescent spermatozoa with a batch of oocytes, followed by activation through the addition of seawater and finally, the incubation of the fertilized eggs in order to promote embryo development and hatching (Yasui et al., 2012). The use of high quality gametes both from males and females during this process is essential in order to achieve both suitable fertilization and hatching rates. In this respect, despite the fact that aquaculture hatcheries have focused more on egg rather than sperm quality (Snook, 2005), some studies have demonstrated that spermatozoa quality has a great influence both on fertilization success and the subsequent development of the embryo and larvae (Butts et al., 2011; Ottesen and Babiak, 2007).

From a practical viewpoint, the sperm quality can be measured by any quantifiable parameter which is directly correlated related to fertilization capacity. Spermatozoa motility has been the most commonly used parameter in evaluating sperm quality (Kime et al., 2001), but other features such as sperm density, plasma composition, head morphology or ATP concentration can be also useful tools to measure sperm quality (Fauvel et al., 2010). The gradual appearance of the computer assisted sperm analysis (CASA) has allowed an objective, rapid and accurate assessment of fish sperm samples, including *T. niphobles* (Gallego et al., 2013a). This kind of software is able to provide a

high number of parameters, which can be related to fertilization ability and thus, to sperm quality. However, despite the fact that there are some studies about the relationship between the percentage of motile cells and fertilization and hatching ability, scarce research has been carried out regarding other sperm kinetic parameters assessed by computerized automatic systems.

On the other hand, the sperm/egg ratio is another essential factor which needs to be taken into account in IVF trials. Usually, an excess of sperm is used in these trials, but an appropriate combination of the number of spermatozoa per oocyte should be used in order to optimize reproductive efficiency in fish farms. Improvements in this area would allow a rational use of gametes, limiting the number of breeding fish in culture stations and reducing production costs (Sanches et al., 2011).

Therefore, the main goals of this study were (i) to analyze the effect of the sperm/egg ratio on the sperm quality parameters assessed by CASA system and (ii) to study the correlations between these sperm parameters and the fertilization and hatching rates in the pufferfish.

2. Materials and methods

2.1 Fish handling and gamete collection

Pufferfish demonstrate a characteristic spawning behavior at Arai Beach near Misaki Marine Biological Station (MMBS, Japan). Large schools of fish arrive to the beach around the new moon at spring tide during the spawning season which occurs between June and July (Yamahira, 1996). Spawning takes place repeatedly from 2 hours before the sunset to that moment, and during that time, male and female pufferfish were caught and moved to the MMBS facilities. Fish were kept in running seawater tanks at 18 °C and all trials were carried out under the approval of the animal guidelines of the University of Tokyo on Animal Care.

Before gamete collection the genital area was cleaned with freshwater and thoroughly dried to avoid the contamination of the samples with faeces, urine or seawater, and gentle abdomen pressure was applied to obtain the gametes both in males and females. Fresh sperm was diluted 1:50 in seminal plasma-like solution (consisting in 130 mM NaCl, 5 mM KCl, 10 mM HEPES and 1 mM $CaCl_2$, pH adjusted to 7.5; (Krasznai et al., 2003)) immediately after its extraction sperm samples were maintained at 4 ºC until motility analysis. Eggs were collected just before the fertilization assay.

2.2 Assessment of sperm quality parameters

Sperm were evaluated in the first hour after extraction mixing 0.5 µl of diluted sperm with 4 µl of artificial seawater (Gallego et al., 2013a). The sperm-seawater mixture was placed and observed in a chamber SpermTrack-10® (Proiser R+D, S.L.; Paterna, Spain). Video sequences were recorded using a high-sensitivity video camera (HAS-220; 50 fps) mounted on a phase contrast microscope (Olympus BX51) with a 10x objective lens (Olympus Splan NH). All the motility analyses were performed by triplicate using the motility module of ISAS (Proiser R+D, S.L.; Paterna, Spain).

The parameters assessed in this study were total motility (TM, %), defined as the percentage of motile cells; progressive motility (PM, %), defined as the percentage of spermatozoa which swim in an essentially straight line; curvilinear velocity (VCL, µm/s), defined as the time/average velocity of a sperm head along its actual curvilinear trajectory; straight line velocity (VSL, µm/s), defined as the time/average velocity of a sperm head along the straight line between its first detected position and its last position; average path velocity (VAP, µm/s), defined as the time/average of sperm head along its spatial average trajectory; the percentage of fast (FA; VAP > 100 µm/s), medium (ME; VAP = 50-100 µm/s) and slow (SL; VAP = 10-50 µm/s) spermatozoa; straightness (STR, %), defined as the linearity of the spatial average path; linearity (LIN, %), defined as the linearity of the curvilinear trajectory; wobble (WOB, %), defined as the trajectory oscillation along its spatial average path; amplitude of lateral head displacement (ALH, µm), defined as the amount of lateral displacement of a sperm head along its spatial average trajectory; and beat cross frequency (BCF, beats/s), defined as the average-time rate at which the curvilinear sperm trajectory crosses its average path trajectory. Some of these kinetic parameters are plotted on Figure 1. Spermatozoa were considered immotile if their VAP was lower than 10 µm/s.

2.3 Experimental setup: fertilization and hatching trials.

This study was divided into two trials as described in Figure 2. In Trial 1, gametes from five males and two females were separately used for fertilizations assays, using 5 of the 10 possible combinations (1 male, 1 female), which were simultaneously assayed in three different sperm/egg ratios (10^3, 10^4 and 10^5 spermatozoa/egg) and using sperm activated after three different times (5, 20 and 40 s).

Eggs from two females were divided into batches of 80-90 eggs and placed into 60 × 15 mm Petri dishes using a micropipette with the tip cut off to prevent compression of the

eggs. A known aliquot of sperm (adjusting the volume according to the calculated sperm/egg ratio) was added to 5 ml of seawater and then the sperm-water solution was added to the corresponding batch of eggs at different post-activation times. After an incubation period of 10 min, the eggs were transferred into a clean Petri dish for incubation with 8 mL of clean seawater. The eggs were then incubated in darkness at a controlled temperature of 20 °C. Fertilization rates were evaluated between 1-2 hrs after insemination by counting the percentage of embryos which reached the 4-cell stage in relation to the total number of eggs used.

In Trial 2, using the 10^4 sperm/egg ratio, new batches of 80-90 eggs from one female were separately fertilized with sperm from five males at different post-activation times (5, 20 and 35 s) with the goal of establishing correlations between sperm quality parameters and fertilization and hatching rates. The gamete collection and the artificial insemination were carried out in the same manner as in Trial 1 and the hatching rates were calculated as the percentage of hatched larvae in relation to the total number of eggs. Dead eggs and larvae were removed and counted when detected during daily inspections, and seawater was exchanged once a day.

2.4 Statistical analysis

The mean and standard error were calculated for all the sperm quality parameters. Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) was used to analyse the data. Significant differences were detected using the Tukey´s multiple range test ($P<0.05$). Pearson´s correlation, coefficient of determination and linear regression analysis were used to find the relationship between the different sperm quality parameters and fertilization/hatching rates. All statistical analyses were performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Effect of sperm/egg ratio and sperm quality parameters on fertilization rates

Sperm quality parameters from samples used in Trial 1 are shown in the Table 1. A time-dependent effect was found, lower values in spermatozoa velocities were registered (VCL, VSL and VAP) the longer the post-activation time. However,

spermatozoa motilities (TM and PM) and other sperm parameters such as FA, LIN, STR, WOB and ALH did not show significant differences until 40 s post-activation. Finally, ME, LE and BFC did not show significant differences over time.

The sperm was used to fertilize egg batches at different post-activation times and with different sperm/egg ratios (Figure 3). When the spermatozoa activated after 5 s were used to fertilize the egg batches, no significant differences were found between the 10^4 and 10^5 ratios (inducing over 94% fertilized eggs in both cases). However, the lowest sperm/egg ratio (10^3) produced significantly lower values (approx. 85%) in comparison to the highest ratios.

Regarding the spermatozoa activated after 20 s, significant differences in fertilization rates were found between the different assayed ratios. While the highest sperm/egg ratio showed the highest fertilization rate (approx. 97%), 10^3 and 10^4 sperm/egg ratios showed significantly lower fertilization rates. Finally, using the sperm activated after 40 s, negligible fertilization rate values were obtained irrespective of the sperm/egg ratio, and even the highest (10^5) showed very low values (3.8%).

3.2 Relationship between the sperm motility parameters and fertilization/hatching rates

Coefficients of correlation (*r*) and determination (*r-squared*) between the sperm motility parameters and fertilization/hatching rates are shown in Table 2. Positive significant correlations between fertilization/hatching rates and several parameters such as TM, PM, FA, VCL, VSL VAP, LIN and WOB were found, although correlation values were slightly lower with hatching rates. The sperm parameters showing the highest positive correlations with fertilization/hatching rates were TM, PM, VCL and VSL, shown in Figure 4 (fertilization rates) and 5 (hatching rates), where a linear regression equation was calculated for each parameter.

Regarding coefficient of determination (*r-squared*; Table 2), which shows the goodness of fit of a model and represents the proportion of variability in a data set that is accounted by the statistical mode, spermatozoa velocities (VCL, VSL and VAP) showed the highest values both in fertilization and hatching rates.

Finally, fertilization and hatching rates showed a high and significant correlation between them (Figure 6).

4. Discussion

Sperm/egg ratio and sperm quality are the main factors affecting fertilization and hatching success in artificial insemination trials (Trippel and Nielson, 1992; Linhart et al., 2008). During the present study, the unavailability of low quality fresh sperm samples forced us to work with spermatozoa at different post-activation times with the aim of mimicking sperm samples of different qualities. Thus, during this study the sperm activated after 5 s represent the high-quality sperm samples (high motility and velocity values) while the sperm activated after 20 (high motility and intermediate velocity values) and 40 s (low motility and velocity values) resemble the medium and low quality sperm samples, respectively. However, it must be noted that the swimming time/speed of sperm activated after 20 and 40 s may be shorter than medium and low quality fresh samples.

In this study we have demonstrated that sperm/egg ratio and sperm quality are factors which are strongly related to each other: when high-quality sperm samples (sperm activated after 5 s) were used in the IVF trials, all the different sperm/egg ratios tested produced high fertilization rates (>80%). However, when lower quality sperm samples (sperm activated after 20 or 40 s) were used, the amount of sperm became an essential element in reaching suitable fertilization rates. These results suggest that both sperm/egg ratio and sperm quality should be taken into account as unique interrelated factors, with it possible to obtain high fertilization rates using a successful combination of high/low quality sperm in high/low volumes.

Nowadays, the aquaculture sector is going through a delicate situation and improvements in some issues with regards to IVF should be carried out (Gallego et al., 2012). In this respect, fixing an appropriate combination of the number of spermatozoa per oocyte seems to be a useful tool to optimize the reproductive efficiency in fish farms. However, little data has been reported in marine species about the optimum sperm/egg ratio, which seems to be a species-specific parameter, finding ratios from 10^3 in Atlantic croacker (*Micropogonias undulatus*, (Gwo et al., 1991)) to 10^6 in northern pike (*Esox lucius*, (Zhang et al., 2011)). In this study, we have demonstrated that sperm/egg ratios from 10^3 to 10^5 can be used to achieve proper fertilization rates in pufferfish. However, the optimal sperm/egg ratio depends on the sperm sample quality, so, when good sperm samples are available, it would be possible to use low sperm/egg ratios ($\leq 10^3$); while when sperm samples with high motilities and velocities values are

253 not available, it would be necessary to use higher sperm/egg ratios ($\geq 10^5$) to achieve
254 high fertilization rates in this species.
255 On the other hand, the use of high quality gametes both from males and females is the
256 other essential factor to reach suitable fertilization and hatching rates both for
257 aquaculture and scientific purposes. With regards to male´s gametes, the percentage of
258 motile cells has been the most used parameter to estimate sperm quality. However, for
259 many years the conventional method of motility evaluation has been subjective, and the
260 current appearance of CASA systems has made it possible to estimate a higher number
261 of sperm parameters by an objective, rapid and accurate technique (Gallego et al.,
262 2013b). In this study we have estimated, for the first time, the relationship between all
263 the parameters provided by a CASA system and the fertilization and hatching rates in a
264 marine fish species. Total motility (TM) and progressive motility (PM) are recognized
265 as important sperm traits for male fertility and sperm competition in fish (Rurangwa et
266 al., 2004). In the present study, high correlations were found between these parameters
267 and FR and HR (r~0.7), the same as occurs in some fish species such as Atlantic halibut
268 (*Hippoglossus hippoglossus*; (Ottesen et al., 2009)), red seabream (*Pagrus major*, (Liu
269 et al., 2007)) or common carp (*Cyprinus carpio*, (Linhart et al., 2000)). However,
270 parallel studies about this relationship in other species have given conflicting results, as
271 negligible correlations were found between TM or PM and fertilization potential
272 (Bozkurt et al., 2006). In this respect, it is worth highlighting that fertilization trials
273 should be carried out both with an optimal sperm/egg ratio and using a wide range of
274 sperm motility values in order not to mask the real correlations between the motility
275 values and the fertilization and hatching rates (Moccia and Munkittrick, 1987).
276 On the other hand, in addition to the percentage of motile spermatozoa as a good tool to
277 predict fertilization ability, spermatozoa velocities may also serve as prognostic
278 indicators of the fertilization potential of sperm (Liljedal et al., 2008). In fact, in our
279 study the highest coefficients of correlation and determination were found for VCL,
280 VSL and VAP, which showed better correlations with FR and HR than the parameters
281 traditionally used to define sperm quality (TM and PM). This result can be explained
282 through logical hypothesis: at the gamete level, the egg-sperm contact could be
283 influenced by several factors such as the amount of spermatozoa, the number of motile
284 spermatozoa, sperm velocity and sperm longevity. When in IVF trials the number of
285 spermatozoa becomes a limiting factor (tight sperm/egg ratio), increases in spermatozoa
286 velocities will enable spermatozoa to look for the egg and penetrate the micropyle at a

faster rate per time unit, increasing in this way fertilization success and thus, fertilization rates (Gage et al., 2004; Linhart et al., 2005). In this respect, the results obtained in Trial 1 using 10^3 and 10^4 sperm/egg ratios support this hypothesis, and sharp decreases in fertilization rates were found when the sperm used showed significantly lower velocities but similar motilities (Fig. 1).

Similar data has been reported in other marine species such as Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*) or green swordtail (*Xiphophorus helleri*), in which spermatozoa velocity seems to be the major component that determines fertilization success and the proportion of the paternity through the sperm competition (Gage et al., 2004; Gasparini et al.; Rudolfsen et al., 2008). In this respect, new approaches in relation to male´s broodstock selection through sperm kinetics features can be used from this perspective. Improvements in the aquaculture sector could optimize the reproductive efficiency in the fish farms, making rational use of gametes possible, limiting the number of breeding fish and, thus, reducing production costs. However, it is important to highlight that breeding fish programs involves a lot of factors and, reducing the number of breeders we could also be decreasing the genetic diversity/basis of broodstock. Therefore, the proper application of several factors among these programs will define the further improvements in aquaculture sector.

Finally, it is important to highlight that besides the sperm/egg ratio and sperm kinetic parameters other factors involved in IVF can modulate fertilization and hatching rates. For example, variations in the spawning environment, oocyte quality, oocyte size or even the micropyle closing time can determine the final results. Therefore, it appears that it is critical to maintain identical fertilization conditions when sperm doses for IVF trials must be estimated, with the aim of avoiding masking effects through the experimental variables (Chereguini et al., 1999).

To sum up, this study showed that both sperm/egg ratios and some sperm kinetic parameters provided by CASA system play a crucial role in the fertilization and hatching success in pufferfish. These kinetic parameters have been defined throughout the IVF trials, and linear regression equations have been developed to the most important parameters with the aim of determining proper values of fertilization and hatching rates. In this respect, spermatozoa velocity seems to be a key factor in this event, especially when the number of spermatozoa per egg is limited in the aqueous environment. This kind of study can serve as a basis for improved efficiency in broodstock management fish reproduction.

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Table legends

Table 1. Sperm quality parameters of sperm samples used in Trial 1 at different post-activation times (5, 20 and 40 s). Data from 5 males of Trial 1 was used and values are expressed as mean ± SEM (n=5). Different letters mean significant differences between post-activation times at the same parameter.
Abbreviations: TM, total motility; PM, progressive motility; FA, fast spermatozoa; ME, medium spermatozoa; SL, slow spermatozoa; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Table 2. Coefficients of correlation (*r*) and determination (*r*-squared) between the sperm motility parameters and fertilization or hatching rates (n=15; data from Trial 2 was used). Asterisks indicates significant correlations between parameters (*, p-*value* < 0.05; **, p-*value* < 0.01).
Abbreviations: TM, total motility; PM, progressive motility; FA, fast spermatozoa; ME, medium spermatozoa; SL, slow spermatozoa; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Figure legends

Figure 1. Schematic diagram of some of the motility parameters recorded by computer assisted sperm analysis (CASA). Black circles represent successive positions of the head of a motile sperm through the video recording. Sperm movement parameters: VCL, curvilinear velocity (μm/s); VAP, averaged path velocity (μm/s); VSL, straight-line velocity (μm/s); ALH, amplitude of lateral head displacement (μm); BCF, beat/cross frequency (beats/s).

Figure 2. Experimental setup for Trial 1 and 2. In Trial 1, gametes from 2 females and 5 males were used using 5 of the 10 possible combinations (n=15), which were assayed in three different sperm/egg ratios and, in each case, using sperm activated after 3 different times (5, 20 and 40 s). In Trial 2, gametes from 1 female and 5 males were used using

all the 5 possible combinations (n=15), which were assayed in a single sperm/egg ratio (10^4) using sperm activated after 3 different times (5, 20 and 35 s). M: male; F: female.

Figure 3. Fertilization rates at different post-activation times and sperm/egg ratios. Data from Trial 1 was used and values are expressed as mean ± SEM (n=5; the experimental unit is each combination sperm/egg ratio and activation time). Different letters mean significant differences between sperm/egg ratios at the same post-activation time.

Figure 4. Relationship between the sperm motility parameters and fertilization rates (n=15; the experimental unit is each Petri Dish). Data from Trial 2 was used to determine the correlations. Linear regression equation was calculated for each parameter.
Abbreviattions: TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity.

Figure 5. Relationship between the sperm quality parameters and hatching rates (n=15; the experimental unit is each Petri Dish). Data from Trial 2 was used to determine the correlations. Linear regression equation was calculated for each parameter.
Abbreviattions: TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity.

Figure 6. Relationship between fertilization and hatching rates (n=15; the experimental unit is each Petri Dish). Data from Trial 2 was used to determine the correlations and linear regression equation was calculated.

Table 1

		Post-activation time		
		5s	**20s**	**40s**
TM	%	80.30 ± 2.06 a	79.39 ± 2.50 a	14.47 ± 2.70 b
PM	%	65.63 ± 1.19 a	70.90 ± 2.46 a	8.00 ± 1.49 b
FA	%	78.04 ± 2.49 a	71.18 ± 6.08 a	3.74 ± 0.86 b
ME	%	1.28 ± 0.33	8.79 ± 4.41	6.90 ± 1.33
SL	%	0.97 ± 0.30	2.42 ± 0.43	3.83 ± 0.83
VCL	µm/s	238.49 ± 1.99 a	152.84 ± 7.43 b	74.83 ± 3.22 c
VSL	µm/s	191.39 ± 2.83 a	133.59 ± 6.94 b	44.86 ± 2.70 c
VAP	µm/s	232.30 ± 1.94 a	151.19 ± 8.03 b	54.72 ± 2.69 c
LIN	%	80.27 ± 1.14 a	87.24 ± 0.75 a	59.93 ± 2.86 b
STR	%	82.37 ± 0.82 a	88.43 ± 0.46 a	71.64 ± 1.49 b
WOB	%	97.42 ± 0.47 a	98.65 ± 0.68 a	73.25 ± 2.48 b
ALH	%	1.53 ± 0.03 a	1.15 ± 0.01 ab	1.08 ± 0.09 b
BFC	beats/s	15.67 ± 0.23	15.10 ± 0.54	14.66 ± 1.61

481 **Table 2**

	Fertilization rate		Hatching rate	
	r	***r*-squared**	***r***	***r*-squared**
TM	0.68**	0.47	0.67**	0.45
PM	0.70**	0.49	0.68**	0.47
FA	0.75**	0.56	0.74**	0.54
ME	-0.80**	0.64	-0.79**	0.63
SL	-0.49	0.24	-0.48	0.23
VCL	0.82**	0.68	0.81**	0.66
VSL	0.80**	0.63	0.78**	0.61
VAP	0.81**	0.65	0.80**	0.63
LIN	0.52*	0.27	0.51	0.26
STR	0.16	0.02	0.14	0.02
WOB	0.59*	0.34	0.57*	0.33
ALH	-0.19	0.04	-0.18	0.03
BFC	-0.44	0.19	-0.42	0.18

Figure 1

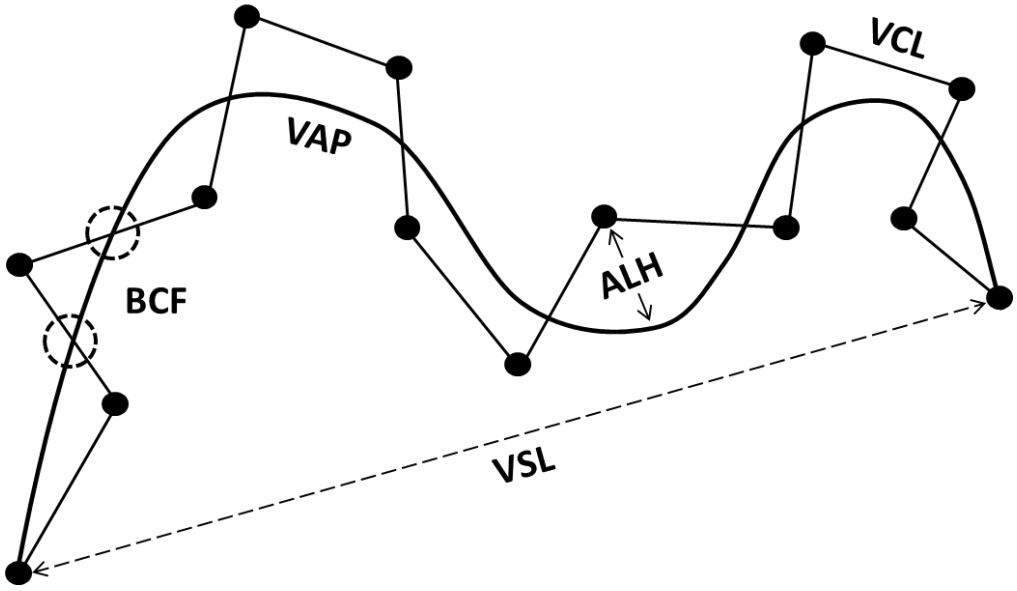


Figure 2

TRIAL 1									TRIAL 2		
Sperm/egg (10^3)			Sperm/egg (10^4)			Sperm/egg (10^5)			Sperm/egg (10^4)		
5s	20s	40s	5s	20s	40s	5s	20s	40s	5s	20s	35s
M1xF1	M1xF1	M1xF1	M1xF1	M1xF1	M1xF1	M1xF1	M1xF1	M1xF1	M6xF3	M6xF3	M6xF3
M2xF1	M2xF1	M2xF1	M2xF1	M2xF1	M2xF1	M2xF1	M2xF1	M2xF1	M7xF3	M7xF3	M7xF3
M3xF1	M3xF1	M3xF1	M3xF1	M3xF1	M3xF1	M3xF1	M3xF1	M3xF1	M8xF3	M8xF3	M8xF3
M4xF2	M4xF2	M4xF2	M4xF2	M4xF2	M4xF2	M4xF2	M4xF2	M4xF2	M9xF3	M9xF3	M9xF3
M5xF2	M5xF2	M5xF2	M5xF2	M5xF2	M5xF2	M5xF2	M5xF2	M5xF2	M10xF3	M10xF3	M10xF3

Figure 3

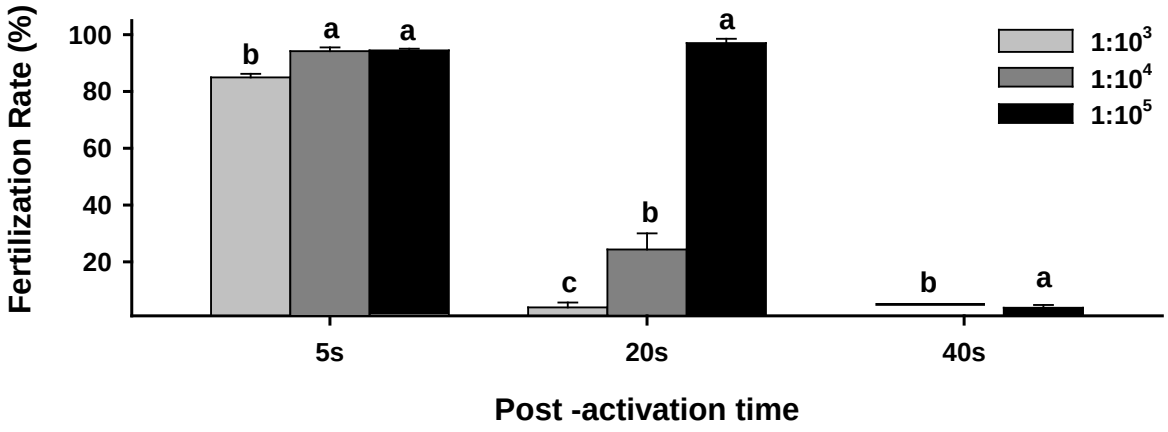
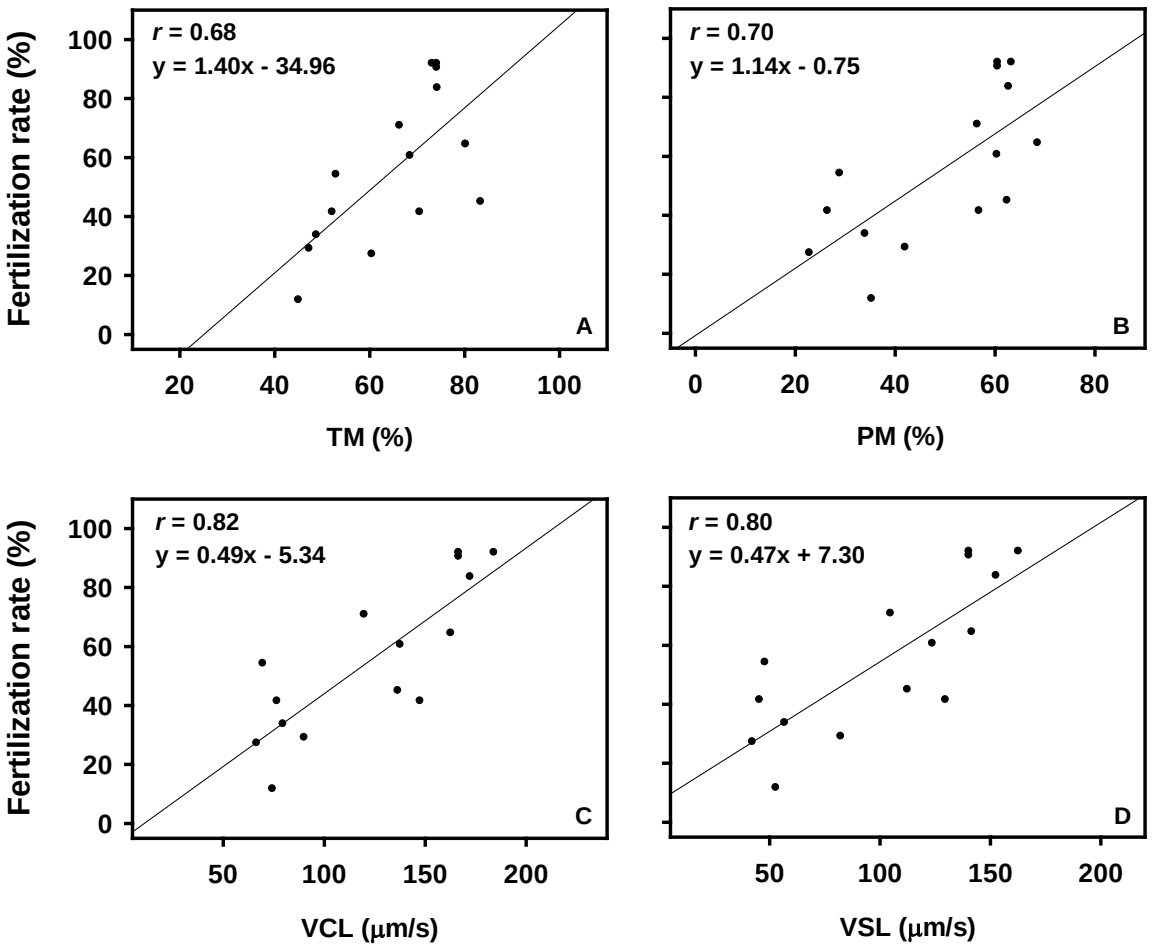
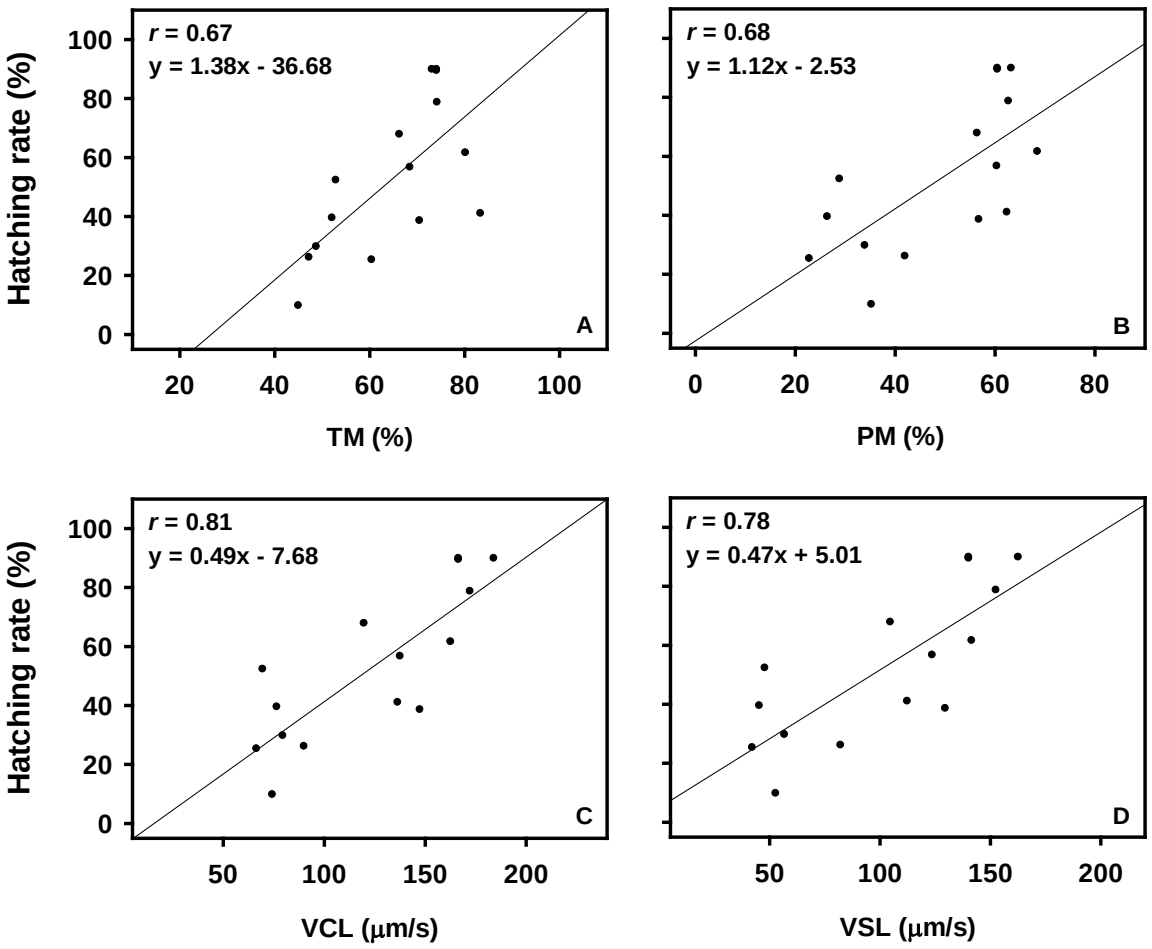

Fertilization Rate (%)
100
80
60
40
20
b
a
a
c
b
a
b
a
1:10^3
1:10^4
1:10^5
5s
20s
40s
Post -activation time

Figure 4

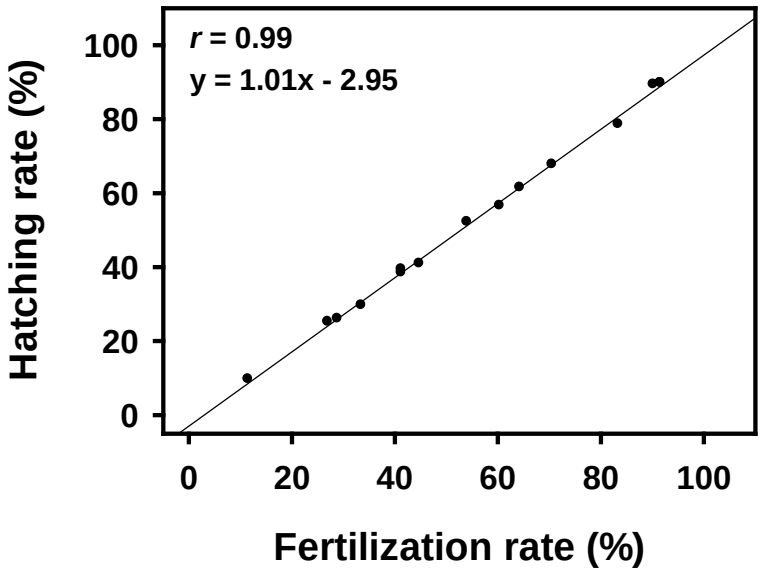

Fertilization rate (%)
r = 0.68
y = 1.40x - 34.96
A
TM (%)
r = 0.70
y = 1.14x - 0.75
B
PM (%)
r = 0.82
y = 0.49x - 5.34
C
VCL (μm/s)
r = 0.80
y = 0.47x + 7.30
D
VSL (μm/s)

492 **Figure 5**



493
494

Figure 6


r = 0.99
y = 1.01x - 2.95
Hatching rate (%)
Fertilization rate (%)
0
20
40
60
80
100