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PII: S0044-8486(17)31400-X
DOI: doi:10.1016/j.aquaculture.2017.11.004
Reference: AQUA 632902

To appear in: *aquaculture*

Received date: 12 July 2017
Revised date: 1 November 2017
Accepted date: 2 November 2017

Please cite this article as: José Beirão, Oddvar H. Ottesen , Optimization of a fertilization protocol for spotted wolffish (Anarhichas minor). The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Aqua(2017), doi:10.1016/j.aquaculture.2017.11.004

Title: Optimization of a fertilization protocol for spotted wolffish (*Anarhichas minor*)

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ABSTRACT

Spotted wolffish, *Anarhichas minor*, is considered a potential species for marine cold water aquaculture diversification. Some biological bottlenecks, including broodstock rearing procedures, hamper the full commercial production of this species. Peculiarities of their reproduction, as the putative internal fertilization and the sperm that is slow moving and motile on stripping, make this species different from most other cultivated species. In this work, we describe practical ways to improve the *A. minor* fertilization protocols through ameliorate usage of the limiting amount of sperm frequently reported in this species. This was achieved by 1) establishing a methodology to accurately measure the sperm concentration; 2) determining the optimal sperm to egg ratio, and; 3) adjusting the gametes contact time accordingly to the used sperm to egg ratio. In the first trial, sperm concentration was measured by direct counting in a haemocytometer; by spectrophotometry and; with the spermatocrit. The absorbance values in a spectrophotometer set at 300 nm after pre-diluting the sperm sample in an extender 100 times had the strongest correlation (R^2= 0.9185) with the haemocytometer cell-counting. In the second trial,

the eggs were fertilized, using the following six egg to sperm ratios: $5x10^3$, $1x10^4$, $5x10^4$, $1x10^5$, $5x10^5$ and $1x10^6$. There were higher fertilization rates, above 90%, when 5×10^5 and 1×10^6 sperm per egg were used with a gametes contact time of 2h. Finally, in the third trial, eight combinations of contact times (1h, 2h, 4h and 6h) and sperm to egg ratio ($5x10^3$, $5x10^4$ and $5x10^5$) were tested. Increased gametes contact time, 6h vs 2h, partially compensated for the lower sperm volumes when $5x10^3$ and $5x10^4$ sperm to egg ratios were used. In addition to the standardization of the fertilization protocols for the *A. minor*, the information in this work is also valuable for other aspects of broodstocks management, and contributes to the understanding of the reproductive biology of this species.

Keywords: sperm concentration; spectrophotometry; spermatocrit; sperm to egg ratio; artificial fertilization; gametes contact time

1. Introduction

Spotted wolffish (*Anarhichas minor*) since the late 90s has been considered a potential species for the aquaculture diversification in countries such as Norway, Iceland or Canada. In Norway there has been some commercial production since the early 2000's, instable around 100 tonnes/year, whereas in Iceland and Canada only pilot-scale production has been tested (Foss and Sparboe, 2009; Le François et al., 2010). In addition to its rich and tasty filets, *A. minor* presents relatively fast growth rates in captivity, reaching 3 kg in 3 years, and the marked value of the product has already been proved (see Foss et al., 2004; Le François et al., 2010 and references within). Nonetheless, competition with other emerging species such as cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*) has halted its development. Recently, there has been an increasing interest from private companies to invest and establish culture of *A. minor* on both sides of the Atlantic. Research efforts have been initiated to solve the remaining biological bottlenecks that still exist for commercial production of *A. minor*, which include broodstock rearing procedures. Similar to what happens to other cultivated fish species, *A. minor* reproductive behaviour is disrupted in captivity. Consequently, males do not display normal spawning behaviour and females release unfertilized eggs, and fertilization must be done artificially *in vitro* (Foss et al., 2004; Kime and Tveiten, 2002; Le François et al., 2010). Nevertheless, several particularities of their reproduction make this species different from most other cultivated species. For instance, the putative internal fertilization (Le François and Archer, 2007), the short supply of sperm, that is motile on stripping and inactivated by seawater, slow moving and very diluted (Kime and Tveiten, 2002), or the long period of incubation up to 1 000 day degrees of the large benthonic eggs, very labour intensive (Sund and Falk-Petersen, 2005). All this together imply that there is a need for a different approach to the fertilization protocols in

this species, which includes dry fertilization and gametes contact time of several hours (Le François and Archer, 2007). As an example, in the close relative common wolffish (*A. lupus*), the best sperm to egg ratio is 2 x 10^5 with gametes contact time up to 7h (Pavlov, 94).

There is a limiting amount of good quality gametes in *A. minor* (Kime and Tveiten, 2002). Nonetheless, due to the lack of knowledge on the optimal sperm to egg ratio, excess of sperm is normally used for the fertilization (Le François and Archer, 2007). However, above a certain limit there is no benefit of using higher proportion of sperm per eggs (e.g. Butts et al., 2014). Additionally, for species where sperm remains motile for longer periods the contact time is also crucial, and longer contact times could compensate for suboptimal sperm to egg ratio. *A. minor* sperm is motile for at least 2 days and retains the sperm swimming parameters values for a few hours after collection (Kime and Tveiten, 2002). As explained by Butts et al. (2009), the longer the contact time the higher the chances of successful encounters between the gametes. In this context, for the development of artificial fertilization protocols, sperm concentration should be accurately measured. Furthermore, sperm concentration is a key parameter to evaluate males status (e.g. Beirão et al., 2011) or to optimize sperm storage protocols (Leclercq et al., 2014). Traditionally, sperm concentration has been measured directly by counting in the microscope, using cell counting chambers, haemocytometer, which is tedious and time consuming (Fauvel et al., 2010). Thus, methods of indirect measurement such as spectrophotometry (e.g. Leclercq et al., 2014) or spermatocrit, that is the percentage ratio of packed sperm in the total volume of semen after centrifugation (Sørensen et al., 2013), are frequently preferred.

In this work we aimed to develop a step by step protocol for artificial fertilization in *A. minor* that maximizes the use of the limited volume amount of gametes, particularly semen. For this, we optimized different steps of the protocol to 1) establish a methodology to easily, and accurately,

measure the sperm concentration; 2) determine the optimal sperm to egg ratio to avoid using excess sperm and; 3) define the best gametes contact time accordingly to the used sperm to egg ratio.

2. Materials and methods

2.1. Broodstock rearing conditions and gametes collection

Semen and eggs (oocytes) were obtained from farmed *A. minor* broodstock kept at the Mørkvedbukta research station, Nord University, Norway in the 2015/2016 and 2016/2017 spawning seasons. The broodstock, mixed age between 8 and 15 years old (males 5.96 ± 0.32kg and 74 ± 1cm, females 5.37 ± 0.49kg and 72 ± 2cm), was kept in a density of approximately 15kg/m2 and a sex ratio 1:1 in square fiberglass tanks of 2 by 2m and water depth of 0.4 m. Fish were fed a commercial feed for marine broodstock twice a week and were exposed to natural photoperiod, and ranging temperature and salinity according to Mørkvedbukta bay natural patterns. The oxygen level was kept always above 80%.

In no instance were the males or females used in these trials hormonally induced. Both males and females in this species mature and produce gametes in captivity during the spawning season without the need for hormonal induction. For semen collection, an initial massage in the abdominal area allowed to release most urine and faeces. Afterwards, semen was collected by lateral massage pressing the testis region and using a slight suction with a Pasteur pipette in the urogenital region. Samples that present a yellowish colour, some deposit and urine crystals or blood were discarded. Only samples with motility higher than 60%, measured visually by counting, were selected. Eggs were collected from females that present the normal “close to

spawning signs", following the procedures described by Le François and Archer (2007). Only egg batches that present a homogeneous colour and normal appearance were used. In four attempts (total of 12), the fertilization values were all below 20% and this raised questions on the eggs quality, so the data were discarded. Gametes were kept in the dark and in a plastic rack in ice (≈ 4ºC) during all the time. *A. minor* eggs are sensitive to light (Le François and Archer, 2007), thus during eggs manipulation a head flashlight with red light was used. Fertilizations were always conducted within 6 hours of eggs collection.

2.2. Trial 1 - Sperm concentration measurement

Concentration of sperm samples of individual males (n=15) was evaluated by 1) direct counting in a haemocytometer; 2) spectrophotometry and; 3) measuring spermatocrit.

For the direct counting, a haemocytometer, Bürker cell-counting chamber, was used. The samples were vortex and pre-diluted 100 times in an extender developed by Kime and Tveiten (2002) (145mM NaCl, 4.55mM $CaCl_2$, 4.83mM $KHCO_3$, 2.37mM $MgSO_4$ and 1mM glucose) and a drop (20µl) placed in one of the chambers mounted with a coverslip. The cells in the four diagonal squares in the large central square were counted, total volume of 0.025mm^3, using a microscope (Leica DM1000) with a 40× lens. Results are expressed in cells ml^{-1}.

In the spectrophotometric reading two sequential dilutions were prepared in the same extender developed by Kime and Tveiten (2002), using 1:9 and 1:99 (semen:extender) after homogenization. Diluted samples (80 µl) were transferred into the disposable cuvettes (Micro UV-Vis, BRAND®). Absorbance values were measured in a spectrophotometer (Genesys 10S UV-Vis, ThermoFisher Scientific) at 260, 300, 350, 400, 500 and 600nm. The 260nm value was

selected because it corresponds to the DNA optical spectrum as explained by Rouxel et al. (2008), whereas the remaining wavelengths were selected to cover the visible spectrum. In each set of readings, a blank with only the extender was always used.

The spermatocrit in the samples was measured filling hematocrit capillary tubes (Hirschmann, Germany) with 65µl semen, that were sealed with hematocrit-sealing material (Assistent, Germany). The capillary tubes were centrifuged (Heraeus Pico 17, ThermoFisher Scientific) at 8 000g for 10min (previous trials with higher RCF did not result in an improved sperm packing). The spermatocrit was measured with a vernier calliper and the result is presented as percentage of packed cells per total semen volume.

Each reading (cell counting chamber, spectrophotometer and spermatocrit) was conducted three times with three different subsamples of each of the 15 males. The value presented correspond to the mean ± SEM of the triplicates.

2.3. Trial 2 - Sperm to egg ratio

To optimize the best sperm to egg ratio, egg batches were collected from four different females at different days. Each of the four batches of eggs was separated in 12 groups, each group with 50 eggs. The eggs were fertilized, using the following six egg to sperm ratios in duplicate ($5x10^3$, $1x10^4$, $5x10^4$, $1x10^5$, $5x10^5$, $1x10^6$), in separate fertilization boxes (Fig 1).

In *A. minor* gametes synchronization (simultaneous availability of mature eggs and sperm) and limiting amount of good quality sperm represents a challenge. However, we wanted to avoid a male effect in our experiment, thus, a pool of sperm from at least two males was used for fertilization of each batch of eggs of the four females. Sperm concentration was measured in

the Bürker chamber and the 50 eggs of each treatment were fertilized with an adjusted volume of semen according to the treatment sperm to egg ratio. The gametes contact time was 2h and the eggs were mixed every 30min. After this time, approximately 250ml of research station seawater, UV filtered, was added to the fertilization boxes and the eggs let to settle for 1h. Thereafter, the ovarian fluid was carefully collected from the surface with a small beaker and the eggs left incubating at 6ºC. The percentage of fertilized eggs was measured after 22h, counting the number of eggs in the 4-cell stage. The duplicate fertilization of each treatment was averaged and the mean value of the means for each of the four batches is presented ± SEM.

2.4. Trial 3 - Contact time

Three different egg to sperm ratios were selected based on the previous trials results (section 2.3) and the following 8 combinations of contact times and sperm to egg ratio were tested in four different egg batches: $5x10^3$ for 2h, 4h and 6h, $5x10^4$ for 2h, 4h and 6h and $5x10^5$ for 1h and 2h. Each treatment was conducted in duplicate with 50 eggs kept in separate fertilization boxes, in total 16 groups (Fig 1).

The experiment set up is largely the same as the described in section 2.3. When the gametes contact time was higher than 2h, eggs were mixed every 1h after the first 2h. Also in this occasion, the duplicate fertilization for each treatment and batch was averaged and the mean value of the means for the four batches is presented ± SEM.

2.5. Data analysis

All data was analyzed with the software R 3.1.2 (R Development Core Team 2014).

Linear regressions between the sperm concentration measured with the haemocytometer and the spermatocrit and the semen absorbance were tested for significance coefficient of determination (R^2) with the 'lm' function. Significant differences between the different linear regressions was tested with the 'aov' function building a GLM where the method to evaluate sperm concentration, either spermatocrit or spectrophotometry, was a nominal variable and the haemocytometer cell-count a continuous variable.

In both the sperm to egg ratio and contact time trials, the results were analysed with an ANCOVA using the 'aov' function with treatment (sperm to egg ratio and contact time) as the main factor, female as covariate and the percentage fertilization as the response variable.

In the three trials there were significant differences and Duncan's test was used for multiple comparisons using the 'duncan.test' function from the 'agricolae' R package (de Mendiburu 2012). The models assumptions were always checked by plotting the models residuals and using the 'bartlett.test' function to verify the homogeneity of variances. In all instances differences were considered significant for $p<0.05$.

3. Results

The average sperm concentration as measured in the haemocytometer was $1.18 \pm 0.20 \times 10^9$ cells per ml (n=15). There was a significant correlation between the spermatocrit and the haemocytometer cell-count ($t_{13} = 10.63$, $p = 8.8 \times 10^{-8}$) with the $R^2 = 0.8968$ (Fig 2). In addition, the spectrophotometric readings were significantly correlated with the haemocytometer cell-count ($t_{13} \geq 3.82$, $p \leq 0.002$). However, there were significant differences between these linear regressions ($F_{12, 181}=26.39$, $p < 2\times10^{-16}$). Indeed, the linear regression between the haemocytometer cell-count and the spectrophotometric reading at 260nm when the samples were

diluted 1:9 ($R^2 = 0.5289$) was significantly different from the linear regression models with samples diluted 1:99 ($0.8614 \leq R^2 \leq 0.9185$) (Fig 2). The linear regression that gave the best explanation of the variance was obtained with the absorbance values for the wavelength of 300nm after sample dilution 1:99 ($R^2 = 0.9185$, $t_{13} = 12.10$, $p = 1.88 \times 10^{-8}$, $y = 4.1276\ x - 0.2359$), nonetheless the readings within the visible range (400, 500 and 600 nm) also had relatively high coefficients of determination (Fig.3).

There were significant differences in the percentage of fertilized eggs among the different sperm to egg ratios ($F_{5, 39}=54.87$, $p < 2\times10^{-16}$). The post-hoc analysis revealed that there were significant higher fertilization rates, above 90%, when 5×10^5 and 1×10^6 sperm per egg were used compared to all other groups and there were no significant differences using either 5×10^4 or 1×10^5 sperm per egg in the fertilization rates with 66.7 ± 9.1 % and 74.3 ± 6.7% respectively (Fig 4).

When different contact times and sperm to egg ratios were tested, there were also differences in the percentage of fertilized eggs ($F_{7, 53}=22.47$, $p = 8.73\times10^{-14}$). The decrease of the contact time from 2h to 1h did not significantly affect the percentage of fertilized eggs when 5×10^5 sperm per egg were used. On the other hand, the percentage of fertilized eggs could be significantly improved when using 5×10^3 or 5×10^4 sperm per egg with longer contact times, 6h vs 2h (Fig 5). In both the sperm to egg ratio and contact time models, there was a significant effect of the female with $p \leq 5.61\times10^{-7}$.

4. Discussion

This work describes practical ways to improve *A. minor* fertilization protocols through ameliorate usage of the often limiting amount of sperm in this species by: 1) accurate measurement of sperm

concentration with a spectrophotometer; 2) using an optimal sperm to egg ratio that could vary between 5×10^5 and 5×10^4 and; 3) adjusting gametes contact time between 2h and 6h according to the sperm to egg ratio.

From the different tested options the absorbance values in a spectrophotometer set at 300 nm after pre-diluting the sperm sample in an extender 100 times had the strongest correlation (R^2= 0.9185) with the haemocytometer cell-counting. Similar high correlations were obtained in other species between absorbance readings and haemocytometer cell-counting (e.g. Butts et al., 2014; Tan et al., 2010). Interestingly, the best wavelength is species specific. In case of fish species, 300nm was the best for *A. minor* (this study), 350 nm for European eel (*Anguilla anguilla*) (Butts et al., 2014) and 400 nm for Atlantic croaker (*Micropogonias undulates*) (Leclercq et al., 2014) and different fish species (Tan et al., 2010). The reason for the different absorbance peaks between species is likely related with the different sperm cells size, shape and composition, but can also be affected with the pick absorbance of confounding variables like presence of urine or blood, especially when using testicular sperm as mentioned by Tan et al. (2010). At the same time, it is not clear why the sperm dilution in the extender (1:99 vs 1:9) improved the spectrophotometric reading of the *A. minor* sperm. In fact, in *M. undulates* Leclercq et al. (2014) only obtained a negligible absorbance in seminal plasma alone, discarding the possibility of improved reading by increasing sample dilution.

Nonetheless, the use of the spermatocrit could also be acceptable for *A. minor* when spectrophotometry is not available, since a relatively high coefficient of correlation was also obtained (R^2 = 0.8968). Indeed, some works recommended the use of this technique for other fish species such as the snowtrout (*Schizothorax richardsonii*) (Agarwal and Raghuvanshi, 2009). Finally, other authors have shown the feasibility of using other techniques such as flow cytometry

(Nynca et al., 2016). However, as mentioned by Rio-Portilla and Beaumont (2008) the use of a spectrophotometer is relatively inexpensive (compared with flow cytometry), fast (compared with the time-consuming haemocytometer cell-counting) and relatively easy to operate in an aquaculture facility.

It is also worth noting that the sperm density obtained in this work is higher (in the range of 10^9) than previously described for *A. minor* (in the range of 10^6) by Kime and Tveiten (2002). This fact could be related with sampling methodology previously described by different authors (Kime and Tveiten, 2002; Le François et al., 2008) that apparently collected semen directly by pressing the abdominal region, instead of the lateral abdominal region where the testis are located. Nonetheless, other factors such as broodstock origin or nutrition could have also affected the sperm density.

Previous studies in *A. minor* reproduction have recommended the use of excess of sperm without specifying the amount (Le François and Archer, 2007). According to our results, when using gametes contact time of 2h a sperm to egg ratio of at least 5×10^5 should be used. This value is comparable to the recommended value for the *A. lupus* of at least 2×10^5 (Moksness and Pavlov, 1996). For comparison, the values recommended for other fish species are quite variable from a few thousands in turbot (*Scophthalmus maximus*) to millions in herring (*Clupea harengus*) (see Butts et al., 2012 and references within). From the aquaculture perspective, different practical aspects can influence the best sperm to egg ratio. As an example, the use of dry vs wet fertilization is sometimes assayed in artificial fertilization (Chereguini et al., 1999). In the case of *A. minor* the selection of dry fertilization is an obvious choice, since the sperm is already motile (Kime and Tveiten, 2002) and the addition of a fertilizing solution will dilute the gametes. Nonetheless, in most external fertilizing species, water facilitates sperm motility activation (e.g.

Chereguini et al., 1999; Sanches et al., 2016) and often abundant water is preferable to a low volume of activation water that negatively affect fertilization rate. In addition, as studied by Gallego et al. (2013) the sperm quality will also effect this ratio. Thus, seasonal variations during the spawning season (e.g. Beirão et al., 2011), inter-individual variability (e.g. Ottesen and Babiak, 2007) or different sperm origins such as stripped vs testis sperm or fresh vs cryopreserved sperm (e.g. Dietrich et al., 2016; Ottesen et al., 2012) should be taken into account. Another important aspect is the gametes contact time that will affect the success of the sperm to encounter an egg, as explained by Butts et al. (2014).

The results of the present work have shown that the increase in the gametes contact time can partially compensate for the lower sperm volume, frequently reported for this species (Kime and Tveiten, 2002; Le François et al., 2008). Indeed, to solve this limitation, contact times up to six hours have been recommended (Le François and Archer, 2007), whereas for *A. lupus* Moksness and Pavlov (1996) recommend between 2 and 7h contact time, dependent on the available sperm volume. Nonetheless, the extremely long contact times represent a limitation to the producers because it implies increased labor. The unusual long contact time needed for this species is certainly related with the slow sperm speed (around 50 µm/s) and zig-zag movement of the sperm described by Kime and Tveiten (2002). For example, the longer contact times tested for other species were 30 min in Atlantic cod (*Gadus morhua*) (Butts et al., 2009), that has a sperm velocity above 120 µm/s (Beirão et al., 2014), and around 3 min in *S. maximus* (Suquet et al., 1995), that has a sperm velocity around 220 µm/s (Cosson et al., 2008). Moreover, the option of using longer contact time should not be ruled out when low volumes of sperm are available. Nonetheless, in the case of *A. lupus*, Moksness and Pavlov (1996) described that contact times longer than 7h have a negative effect on the fertilization success. Overall, several aspects of the

species reproductive biology likely affect the optimal sperm to egg ratio and contact time, and this should be considered when adjusting the fertilization protocols. For example, Stockley et al. (1996) sustains that sperm characteristics such as volume and longevity are related to the females reproductive biology features such as internal vs external fertilizers. In this context, the ejaculate in the external environment will be dispersed rapidly and exposed to a hostile environment and thus shorter contact times (lower sperm longevity) closely mimic the natural conditions. That being said, it was not this work objective to discuss if *A. minor* is either an internal or external fertilizer.

Finally, there was a significant effect of the female in the fertilization results. It is not clear if this effect was directly related with the females or exclusively with the egg batch since each female only contribute with one batch for the experiment. As mentioned by Dietrich et al. (2012), several factors can affect egg quality such as genetics, nutrition, stress, health status, water temperature, handling, and time passed after egg ovulation. Unfortunately, in *A. minor,* only initial approaches were taken that focused mainly on fertilized eggs quality (e.g. Tveiten et al. 2004, Desrosiers et al. 2008). Thus, future studies should try to establish egg (oocytes) quality parameters prior to fertilization for this species. On the other hand, a male effect in this study can not be completely discarded, but is unlikely because in all instances we used a pool of sperm from at least two males.

In addition to the importance of the standardization of the fertilization protocols for the *A. minor* aquaculture, this work results will also be valuable for other aspects of the broodstocks management, such as, increasing the effectiveness of sperm cryopreservation protocols by standardizing the number of cells in each cryogenic sperm straw (see previous studies in *A. minor* sperm cryopreservation by Gunnarsson et al. 2009; Le François et al., 2008). Moreover, these results also contribute to understand this species reproductive biology, which is currently

designated by the Committee on the Status of Endangered Wildlife in Canada as 'threatened' in north-west Atlantic Ocean (COSEWIC, 2012).

According to the present study, we recommend to measure the *A. minor* sperm concentration by spectrophotometry. Furthermore, for an average of 5 000 eggs per L we recommend to use at least 2.5×10^9 sperm per L of eggs for a 2 h gamete contact time. Therefore, for an average sperm concentration of 1.17×10^9 sperm per ml we recommend to strip at least 2.15 ml of good quality sperm per L of eggs collected. In the event a lower sperm volume is available, this could be compensate by using at least 2.5×10^8 sperm per L of eggs and a contact time of 6h.

Acknowledgements

This work was partially supported by the Nordland county (project nr. 2015-0737). The authors would like to thank to overingeniør Bjørnar Eggen, Nord University, for fish maintenance and support during sampling as well as João Santana for assistance during some experiments. AMINOR AS is the owner of the broodstock from which the gametes were obtained.

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Fig. 1. Schematic representation of the experimental design for the sperm to egg ratio trial and the contact time trial. The dashed line represent treatments only for the contact time trial. The top row represents the 4 batches of eggs collected on different days from different females, the middle row represents the 6 or 8 treatments for the sperm to egg ratio trial and contact time trial respectively, the third row the treatment details, and the bottom row represents the technical replicates used in each egg batch and treatment.

Fig. 2. Linear regression between direct cell-counting with a Bürker chamber and the percentage of spermatocrit (n=15).

Fig. 3. Coefficient of determination (R^2) values obtained from the linear regressions between the direct cell-countings and the absorbance values obtained with the different wavelength in the spectrophometer (260, 300, 350, 400, 500 and 600 nm) for the two semen:extender dilutions 1:9 (solid line) and 1:99 (dashed line).

Fig. 4. Percentage of fertilized eggs in spotted wolfish relatively to the sperm to egg ratio used. Different letters stand for significant differences detected with Duncan's multiple comparison test ($p<0.05$). The error bars represent the standard error of the mean (n=4).

Fig. 5. Percentage of fertilized eggs in spotted wolfish relatively to the contact time and the sperm to egg ratio used. Bars with stripes for 5×10^3 sperm per egg, dark grey bars for 5×10^4

sperm per egg and light grey bars for 5×10^5 sperm per egg. Different letters stand for significant differences detected with Duncan's multiple comparison test ($p<0.05$). The error bars represent the standard error of the mean (n=4).

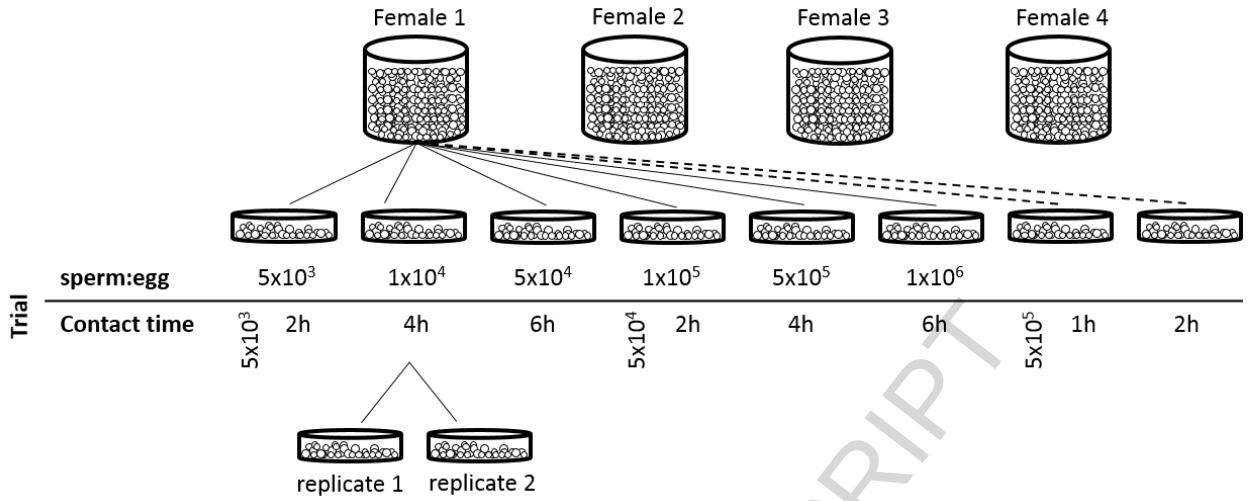

Figure 1

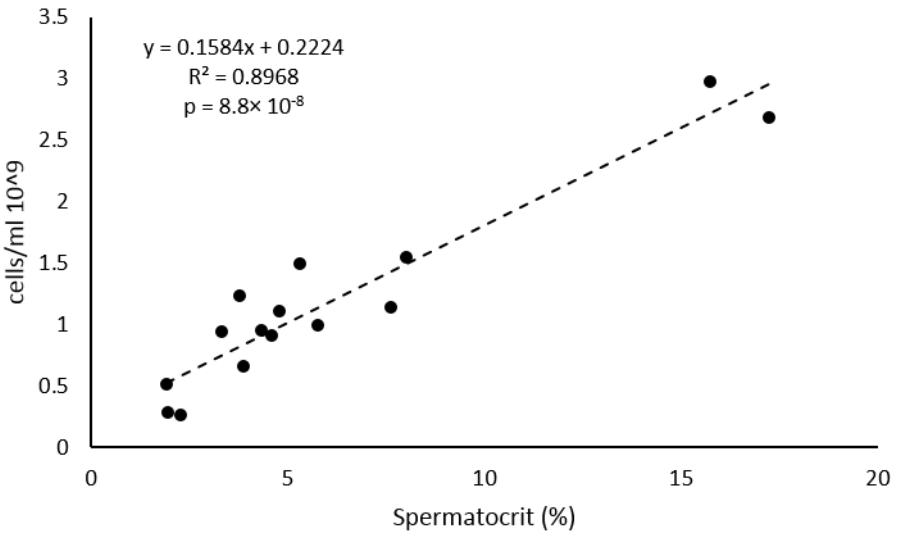

Figure 2

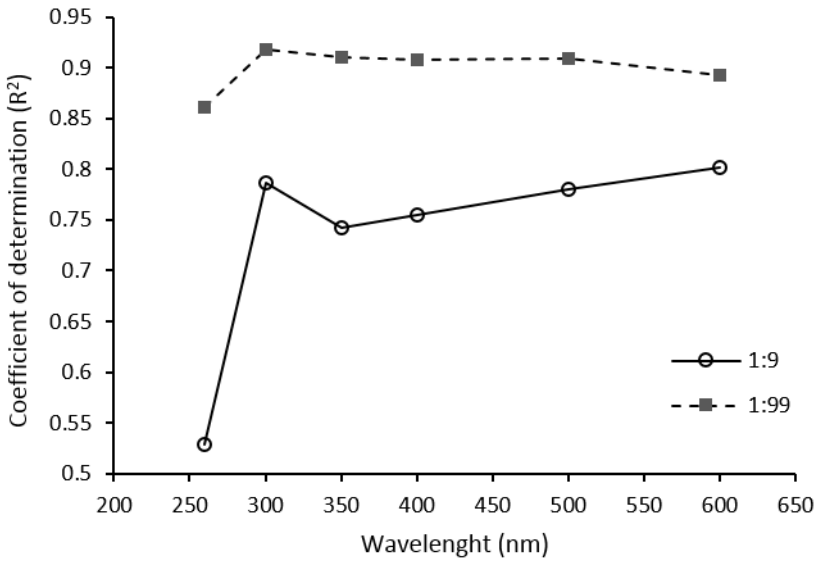

Figure 3

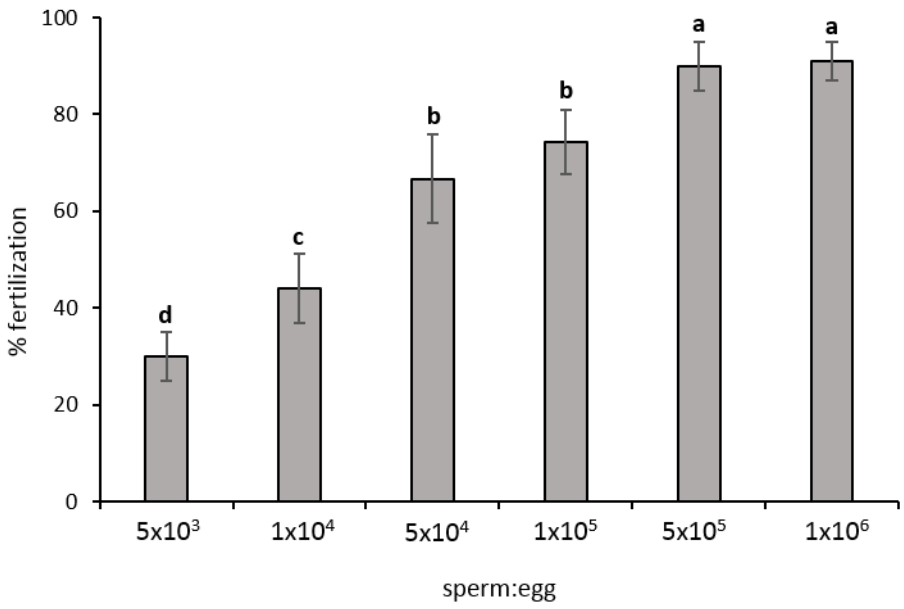

Figure 4

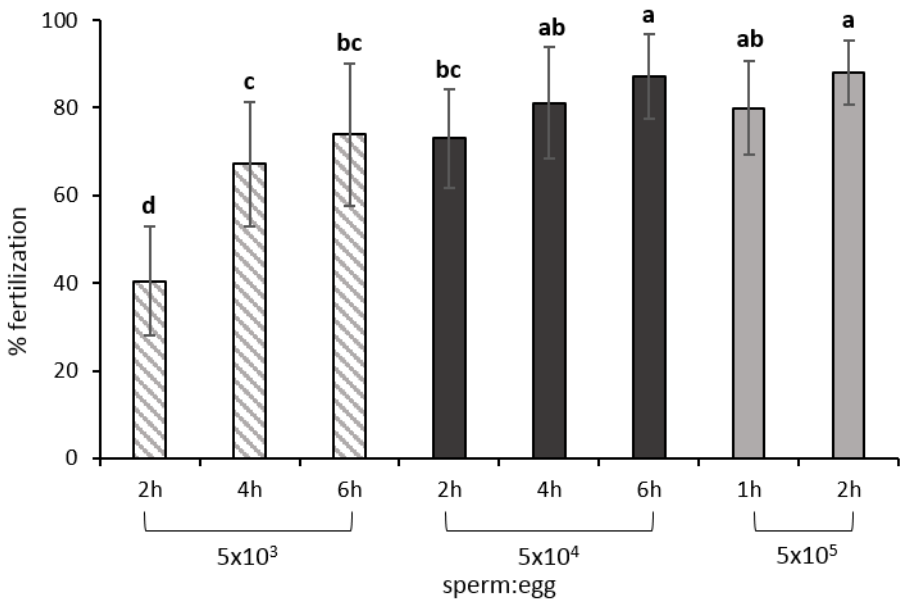

Figure 5

Highlights

- Spotted wolffish sperm concentration can be accurately measured in a spectrophotometer set at 300 nm after pre-diluting the sperm sample in an extender 100 times.
- When using gametes contact time of 2h a sperm to egg ratio of at least 5×10^5 should be used in spotted wolffish.
- An increased contact time (6h vs 2h) could be used to compensate for lower volumes of stripped sperm in spotted wolffish.