GAMETE QUALITY AND MANAGEMENT FOR *IN VITRO* FERTILISATION IN MEAGRE (*Argyrosomus regius*) TO FACILITATE THE IMPLEMENTATION OF GENETIC BREEDING PROGRAMS.

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Introduction

The meagre (*Argyrosomus regius*) has good aquaculture potential due to good market price, fast growth, low feed conversion ratio, relatively easy larval rearing and established induced spawning protocols (Duncan et al., 2013). A bottleneck to the expansion of the industrial production of meagre is that many producers have breeders from few populations and few families from these populations, which indicates that there is a need for well-designed breeding programs to avoid problems related to inbreeding. The present study aimed to define protocols for obtaining gametes of high quality and for *in vitro* fertilisation to facilitate the implementation of genetic breeding programs.

Materials and Methods

The meagre broodstock (mixed wild and cultured) was held under a natural photo-thermal regimen in two 70m³ circular tanks in recirculation (IRTAMar®). During the experiment, the temperature was maintained at 18.6ºC. Maturity was determined from ovarian biopsies and ease to express sperm. To obtain sperm, an injection of GnRHa (15 µg/kg) was applied to males (n=5, mean weight 16 ± 3 kg) 24h before sperm collection. Sperm was obtained before ovulated eggs, diluted 1:4 in modified Leibovitz cell culture medium and stored over ice. Sperm quality and concentration was assessed with ImageJ CASA. To obtain eggs, females (n=14, mean weight 20 ± 6 kg) with oocyte diameter >550µm were induced to ovulate with a single GnRHa injection (15µg/kg). The injections were applied at 20:00-22:00h and the females held separate from males in darkness. From 35 to 45h post GnRHa injection the females were checked for ovulation every 2.5h. Batches of eggs were stripped when ovulation was detected and during subsequent checks to have a time series of egg quality of eggs retained in the ovarian cavity after ovulation. Once ovulated eggs were obtained, *in vitro* fertilisation was made immediately by adding sperm and seawater to the eggs at the same time. Egg quality was assessed by determining the percentage of developing eggs. Different sperm:egg ratios were tested from approximately 3,000 to 500,000 sperm egg^{-1} to determine sperm requirements for *in vitro* fertilisation.

Results

Ovulated eggs from 13 inductions were observed from 35h onwards (Fig. 1). Optimal egg quality was observed at 38-39h after the GnRHa injection. There was a high variability in the fertilisation rate of eggs obtained from 35 to 36h and two groups (bimodal) of egg quality were observed, poor eggs (<20% fertilisation) and good eggs (>70% fertilisation). The poor eggs appeared to be related to incomplete ovulation, as only small volumes of eggs were obtained and fertilisation for these females increased from 35 to 39h. Batches with good quality eggs were fully ovulated eggs that were easily stripped. From 38-39h to 43–44h there was a decline in egg quality that was attributed to over-ripening. Egg quality was maintained for a maximum of 2.5h after ovulation and then deteriorated rapidly over 2.5h (Fig. 1). Sperm quality after collection, was maintained without decline for up to 7h in modified Leibovitz medium and sperm did

not appear to affect fertilisation success. The optimal sperm:egg ratio, in order to obtain high percentage of fertilisation, was 200,000+ sperm egg^{-1}.

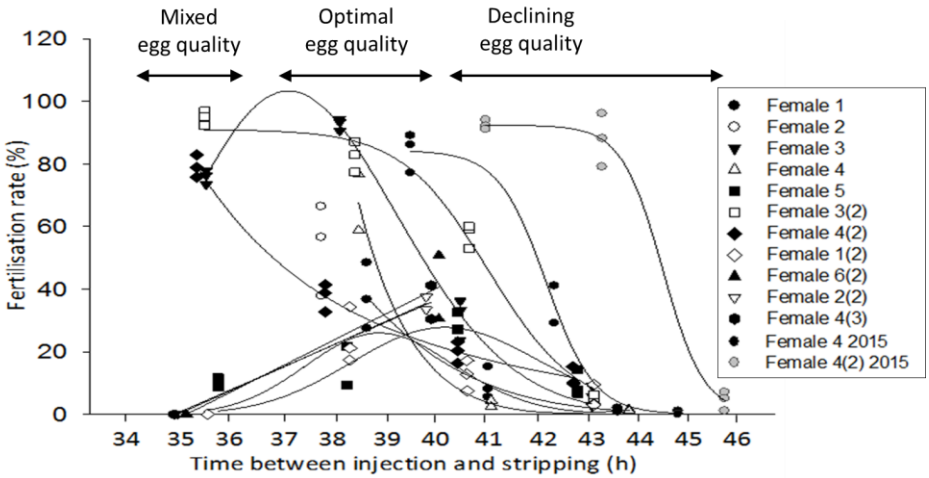

Figure 1. Fertilisation rate (%) of eggs from individual meagre (*Argyrosomus regius*) stripped at different times after a 15µg/kg GnRHa injection. Each line represents an ovulation of an individual female that was stripped sequentially every 2.5 hours. Three periods were proposed: (a) "Mixed egg quality" characterised by poor quality eggs due to partial ovulation and good egg quality eggs from fully ovulated females. (b) "Optimal egg quality" most females had completed ovulation. (c) "Declining egg quality" when eggs were over-ripening. All but two ovulations were from 2016 and the timing of the two ovulations from 2015 was later than the ovulations in 2016.

Discussion and Conclusion

Ovulation time and the short window of optimal egg quality were the critical aspects in the protocol. The variability in ovulation time can be attributed to factors such as temperature, maturity status, individual variability, culture environment and different stocks. Optimal egg quality was characterised by a short window of egg viability of ~2-3h after ovulation. The variability in ovulation time resulted in variation in the timing of the window of egg viability. When eggs were stripped too late after the window, egg quality was poor due to over-ripening and when eggs were stripped too early, egg quality was poor due to partial ovulation. Similar short windows of egg viability have also been observed in Senegalese sole (Rasines et al., 2012). The present study indicated that ovulation and optimal egg quality was achieved 38-39h after GnRHa injection, however, stocks in other hatcheries may show variation and differences should be expected. The sperm:egg ratio was high compared to seabass (Fauvel et al., 1999) although sperm concentration and motility duration were similar. The sperm:egg ratio in this study ensured high fertilisation, but future works may revise the ratio downwards. In conclusion, *in vitro* fertilisation consisted of immediately mixing 40 µL of sperm (1:4 in modified leibovitz = ~3.4 x 10^8 spermatozoa) for 1mL of eggs (~1700 eggs) stripped within 2-3h post ovulation, and immediate activation with a minimum of 0.5mL of seawater mL^{-1} of eggs.

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