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The effect of sperm to egg ratio and gamete contact time on fertilization success in Atlantic cod *Gadus morhua* L.

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ABSTRACT

Currently, Atlantic cod (*Gadus morhua*) is the primary finfish species being developed for aquaculture in North Atlantic waters. Despite the importance of this species, no research has been conducted to assess the effects of sperm density and gamete contact time on egg fertilization. In two separate experiments male and female gametes were crossed using nested factorial designs. For each male–female combination we tested sperm to egg ratios ranging from 1×10^3:1 to 5×10^6:1. We also tested two gamete contact times where sperm and eggs were held in contact with each other for 5 or 30 min. Mixed-model ANOVAs indicated that sperm density and gamete contact time had a significant effect on fertilization success. Below a sperm to egg ratio of 1×10^5:1 fertilization success significantly decreased. Therefore, a standard sperm to egg ratio of 1×10^5 sperm per egg is recommended for fertilization in Atlantic cod. At the 1×10^3:1, 5×10^3:1, and 1×10^4:1 sperm to egg ratios maximum fertilization occurred after 30 min sperm to egg contact time. Gamete contact time was not significant at sperm to egg ratios of 1×10^5:1 and 1×10^6:1. Both the maternal and paternal variance components were significant for fertilization success. This information has important implications for optimizing family production in selective breeding programs, conserving sperm from superior pedigree in genome banks, maximizing the use of available gametes in hatchery or research facilities, and understanding mating success in the wild.

1. Introduction

Historically, Atlantic cod (*Gadus morhua*) has been the most important gadoid species in the North Atlantic from both economic and cultural perspectives (Jensen, 1972; Scott and Scott, 1988; Kurlansky, 1997). Reduced wild-fishery landings, increased market value (Brown et al., 2003), high growth potential (Otterlei et al., 1999), antifreeze protein production (Goddard et al., 1999), and capabilities for grow-out in existing Atlantic salmon, *Salmo salar* L. infrastructure have stimulated interest in commercial culture of this species (Rosenlund and Skretting, 2006).

Artificial fertilization has been routinely used to generate embryos and larvae of Atlantic cod (Kjørsvik and Lønning, 1983; Trippel and Neilson, 1992; Trippel and Morgan, 1994; Litvak and Trippel, 1998; DeGraaf and Berlinsky, 2004; Rideout et al., 2004). Despite the ubiquitous use of this technique there has been no concentrated effort made to assess the effects of sperm density and gamete contact time on egg fertilization success for this species. Presumably, in past studies excess sperm have been used for fertilization. This methodology, however, does not allow for intermale comparisons of fertilization potential as sperm density likely varies across males.

Most research aimed at determining the optimal sperm to egg ratio in fishes has been conducted on freshwater species, for which gamete viability persists for 1–2 min (reviews in Suquet et al., 1994, 1995; Rurangwa et al., 1998). Atlantic cod sperm, on the other hand, remain motile for up to 60 min and can achieve fertilization ranging from 53–85 to 45–57% after being exposed to seawater for 30 or 60 min, respectively (Trippel and Morgan, 1994). The ability of cod eggs to become fertilized after being in seawater has been shown to be highly dependent on egg quality. Davenport et al. (1981) found cod eggs could achieve 50% fertilization success after storage in seawater (5 °C, 34 ppt) for 2 h. Kjørsvik and Lønning (1983) found this level of fertilization was achieved 1 h after egg exposure to seawater (5 °C, 34 ppt), while in poor egg batches within 30 min. Poor egg quality was defined as a slower cortical reaction and slower rise in osmolarity after fertilization (Kjørsvik and Lønning, 1983). Results of these studies suggest that investigators may utilize Atlantic cod's gamete longevity properties in seawater to prolong gamete contact times, which may significantly increase fertilization success.

The present study was conducted to assess the effects of sperm density and gamete contact time on fertilization success of Atlantic cod under controlled conditions. The influence of parental effects on fertilization success was also examined. This information may be used

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to (i) determine the minimum sperm density required to ensure successful fertilization for the aquaculture industry, selective breeding programs, and experimental purposes and (ii) further understand the reproductive biology of wild cod.

2. Materials and methods

2.1. Broodstock collection and management

Broodstock were collected on Georges Bank (41–42° N longitude; 66–67° W latitude) from 12–14 September and 2–3 October 2006 by commercial fishermen using long-line trawl. Upon capture broodstock were transported to the Department of Fisheries and Oceans Biological Station in St. Andrews, N.B., Canada (45° N longitude; 67° W latitude), where they were maintained in a flow-through seawater system under ambient photoperiod and salinity. The broodstock were kept at mean (±SD) dissolved oxygen of 10.4±0.7 mg l^{-1}. Temperature ranged from 12.6 °C in September 2006 to 4 °C in February 2007. Fish were maintained on a diet of frozen squid *sp*. and Atlantic mackerel *Scomber scombrus* L. Broodstock were not fed during the spawning season (December 2006–February 2007) (Fordham and Trippel, 1999).

Fish were sedated with Aquacalm (0.2 g l^{-1}; Syndel International, Vancouver, BC, Canada) prior to anesthesia with MS-222 (Syndel International, Vancouver, BC, Canada) in order to minimize or eliminate the adverse effects experienced by broodstock during handling and stripping of gametes. Sperm were obtained by applying slight pressure on the abdomen, and collecting sperm in 40 ml dry Pyrex beakers. To avoid seawater, urine, and feces contamination the initial male ejaculate was discarded and the external urogenital pore was wiped dry with paper towel. After stripping, sperm samples were held in a 4 °C temperature controlled cold room. For females exhibiting signs of ovulation; such as enlargement of the abdomen, eggs were collected by applying pressure along the abdominal cavity towards the opening of the genital duct. Eggs were collected using dry 1 l polyethylene containers, which was then covered with aluminum foil, and held on crushed ice until use (within 1 h of being expelled from the ovary). All male gametes used for experimentation were crossed within 4 h of collection.

2.2. Estimating sperm and egg density

Sperm density was counted under a compound microscope (Leica DMLB) at 400× magnification using an improved Neubauer haemocytometer. Milt from each male was first diluted 500-fold by adding 10 µl of milt to 5 ml of a sucrose-based diluent (125 mM Sucrose, 100 mM $KHCO_3$, 6.5 mM Reduced glutathione 98%; pH 8.1, 4 °C) that does not activate Atlantic cod sperm (Rideout et al., 2004). To obtain homogenous milt-diluent solutions samples were mixed on a vortex mixer for 10 s. Duplicate dilutions were made for each male sample. For each dilution three counts of five squares (1 mm^2) were inspected. The mean of the three counts for each dilution was calculated, and then the mean of these two values was used to determine the actual sperm density ml^{-1} per male (Tvedt et al., 2001).

Immediately after stripping, the numbers of eggs ml^{-1} from each female were determined by counting five 0.2 ml random subsamples under a dissecting microscope (Leica MZ95) at 8× magnification. The average of the five subsamples was used to calculate the number of eggs ml^{-1}.

2.3. Artificial fertilization

All instruments used for fertilization were chilled at 4 °C prior to use. Eggs were collected and placed into 100 mm×15 mm Petri dishes. Approximately 200 eggs (mean±SE was 197±2.2 eggs) were placed into each dish using a 1.0 ml syringe. The tip of each syringe was cut off to prevent the eggs from being compressed. Using a micropipette a known volume of sperm (adjusted according to the calculated sperm density of each male) was added to 200 ml of seawater and then stirred with a sterile glass rod for 5 s. Salinity of the seawater was 31 ppt. The sperm-seawater solution (40 ml) was then added to the eggs in each Petri dish. After the experimental gamete contact time, excess sperm were rinsed through a net (350 µm mesh size) with an ample quantity of UV sterilized seawater. After rinsing, eggs were transferred into a clean Petri dish for incubation. The embryos were incubated inside a temperature-controlled room at 4 °C until being examined for fertilization success.

2.4. Experiment 1: determination of the optimal sperm to egg ratio

Sperm were obtained from 12 adult males. Eggs were collected from three females. Eggs from female 1 were crossed with milt from males 1–4, eggs from female 2 were crossed with milt from males 5–8, and eggs from female 3 were crossed with milt from males 9–12. For each male–female combination we tested 8 sperm to egg ratios; 1×10^3:1, 5×10^3:1, 1×10^4:1, 5×10^4:1, 1×10^5:1, 5×10^5:1, 1×10^6:1, and 5×10^6:1. Four replicate crosses were completed for each sperm to egg ratio for a total of 384 crosses for the three females (12 males×8 sperm to egg ratios×4 replicate crosses). Gametes were held in contact for 5 min before excess sperm were removed by rinsing with seawater.

2.5. Experiment 2: effect of gamete contact time

Sperm were obtained from 11 adult males. Eggs were collected from three females. Eggs from female 4 were crossed with milt from males 13–16, eggs from female 5 were crossed with milt from males 17–19, and eggs from female 6 were crossed with milt from males 20–23. For each male–female combination we tested two gamete contact times where sperm and eggs were held in contact with each other for 5 or 30 min. For each gamete contact time we tested 5 sperm to egg ratios; 1×10^3:1, 5×10^3:1, 1×10^4:1, 1×10^5:1, and 1×10^6:1. Four replicate crosses were completed for each sperm to egg ratio for a total of 440 crosses for the 3 females (11 males×2 gamete contact times×5 sperm to egg ratios×4 replicate crosses).

2.6. Data collection

2.6.1. Percent motile sperm

The percent of motile sperm per male was estimated by adding 0.1 µl of semen onto the center of a hemacytometer grid, situated on a temperature controlled microscope stage (20/20 Technology Inc., Wilmington, NC), maintained at 4 °C. The sperm were activated for motility by adding 40 µl of seawater (4 °C, salinity was 31 ppt) followed by the addition of a coverslip. Motility was tested 10–15 s after the addition of seawater. Sperm motilities were observed and recorded using a compound microscope (Leica DMLB; 400× magnification) equipped with a Panasonic CCTV camera (WV-BD400), time–date generator (WJ-810), monitor (WV-5410), and video cassette recorder (AG-5700). The percent of sperm cells exhibiting progressive forward motion was assessed by placing a 21.5×28 cm acetate sheet over a computer monitor during playback. The acetate sheet was divided equally into four squares. The video image was paused and the location of 30 sperm cells in each of the four squares was marked on the acetate. The tape was advanced 10 frames and the number of motile sperm was recorded (Litvak and Trippel, 1998). Three replicate motility trials were conducted for each male. The mean of the four squares per replicate was used for statistical analysis.

2.6.2. Fertilization success

Fertilization success was determined 20–30 h post-fertilization, by examining a minimum of 150 eggs per replicate. Embryos were observed and images captured using a dissecting microscope (Leica MZ95) equipped with a digital camera (Q Imaging MicroPublisher