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Fertilization strategies for winter flounder: effects of spermatozoa density and the duration of gamete receptivity

Ian A. E. Butts[1,*], Paymon Roustaian[2], Matthew K. Litvak[3]

[1]Department of Biological Sciences, University of Windsor, Windsor, Ontario N9B 3P4, Canada
[2]Department of Biology and Centre for Coastal Studies and Aquaculture, University of New Brunswick, Saint John, New Brunswick E2L 4L5, Canada
[3]Department of Biology, Mount Allison University, Sackville, New Brunswick E4L 1G7, Canada

ABSTRACT: Winter flounder is one of the most commonly used models for studying fish biology in North America; however little is known about their reproductive ecology, especially during the spawning event. The objectives of this research were to determine the optimal number of spermatozoa required to fertilize eggs and to explore how long spermatozoa (30 to 240 s post-activation) and eggs (30 to 7680 s post-activation) are receptive to fertilization after exposure to seawater. We conducted experiments using gametes from wild-caught fish and measured fertilization success by examining eggs at 5 to 6 d post-fertilization. On average 34 038 sperm cells per egg were required to fertilize 81.3% of the eggs. Duration after spermatozoa activation had an effect on the proportion of eggs fertilized ($F_{3,6.69}$ = 338.38; $p < 0.0001$; mixed-model ANOVA). At 30 s post-spermatozoa activation, 98% of the eggs were fertilized. After 60 s, a significant decrease in fertilization success was detected. Duration after egg exposure to seawater had an effect on the proportion of eggs fertilized ($F_{8,16}$ = 19.89; $p < 0.0001$; mixed-model ANOVA). For all trials (30 to 1920 s), the percentage of eggs fertilized ranged from 61 to 90%. A significant decrease to 11% occurred at 3840 s after egg exposure. This area of research has particular importance for our understanding of reproductive strategies, evolutionary challenges, reproductive potential and recruitment. In addition, examining sperm–egg interactions provides information important to management of living and frozen-thawed gene banks.

KEY WORDS: Marine fish · Reproduction · Sperm · Egg · Fisheries · Aquaculture · Segmented regression

INTRODUCTION

The number of reproductive adults, quantity of gametes released from spawning fish, spermatozoa and egg quality, as well as fertilization success (among others) determine the reproductive potential of fish stocks (Marshall et al. 1998, Trippel 1999, Marteinsdottir & Begg 2002). Although not studied in great detail, an understanding of gamete interactions is clearly important and should be the starting point for examining reproductive ecology and recruitment potential of wild and cultivated stocks. In particular, we need to know: the number of spermatozoa required to fertilize each egg, the longevity of spermatozoa after exposure to water, and the length of time eggs are receptive to fertilization after release.

Eggs are often viewed as limited in supply. Spermatozoa because of their high numbers are not. However, this is not necessarily the case; spermatozoa for many species of fishes are limited (Rideout & Burton 2000, Almeida et al. 2008). For example, spermatogonial proliferation in Atlantic cod of Norwe-

*Email: iana.e.butts@gmail.com

gian coastal origin starts in August and continues for about 6 months and the number of pre-spawning spermatogonia present in the testes essentially determines the numbers produced each season (Almeida et al. 2008). In addition, since most fishes are external fertilizers and the medium in which the spermatozoa is released can be turbulent, i.e. oceans and rivers (Trippel 2003, Faria et al. 2006), there may not be an 'unlimited' number of spermatozoa available to fertilize eggs released by females in these environments.

Spermatozoa longevity and egg receptivity — the length of time an egg is receptive to be fertilized by a sperm cell — also impact the success of a fertilization event (Kjørsvik & Lønning 1983, Trippel & Morgan 1994, Butts et al. 2009). For instance, if female mate choice is important, then eggs may be receptive for a short period of time to reduce the chance of fertilization by non-desirable males. In contrast, if eggs are receptive for longer periods of time then the probability of fertilization by multiple males should increase; this may be important in situations when the probability of spermatozoa to egg encounters are low (Trippel 2003). If spermatozoa are viable for longer periods of time, then the potential of contacting and fertilizing an egg increases (Butts et al. 2009). However, if there is limited time available to fertilize an egg, then selection should favor faster–swimming spermatozoa. This will most likely be at the expense of longevity because there are limited amounts of energy reserves available for spermatozoa motility (Perchec et al. 1995, Burness et al. 2005, Butts et al. 2010). Ultimately, these gamete strategies provide an evolutionary challenge for fertilization for both males and females, as evidenced by counter-strategies employed. Therefore, an inspection of spermatozoa longevity and egg receptivity provides valuable information on reproductive behavior; i.e. whether a species employs a promiscuous or a non-promiscuous mating system.

Even though winter flounder *Pseudopleuronectes americanus* is one of the most studied marine fishes in North America (626 title citations reported in Web of Science on 13 Jan 2012) little is known about its reproductive ecology. Winter flounder is a benthic right-eyed flatfish (Family Pleuronectidae) distributed throughout the North Atlantic Ocean from Labrador, Canada to Georgia, USA (Bigelow & Schroeder 1953). Winter flounder are primarily found in shallow coastal waters and estuaries although several offshore populations exist, including the Sable Island Bank population, the Western and Browns Bank population on the Scotian Shelf, and the Georges Bank population (McClelland et al. 2005). Spawning occurs from late winter in southern areas to early spring in northern areas (Scott & Scott 1988). Winter flounder are *r*-selected (produce many small eggs), single batch spawners and females produce from 192 240 to 3.33 million eggs per season (Scott & Scott 1988, Buckley et al. 1991). Winter flounder males, like many other fish species, undergo spermatogenesis in the fall (Moulton & Burton 1999) and the spermatozoa produced during gonad maturation is limited to the number of spermatogonia present in the testes of each male. Artificial fertilization has long been used to generate embryos and larvae for winter flounder. High fertilization and hatch success rates have been reported with the use of both fresh and cryopreserved spermatozoa (Rideout et al. 2003, Butts & Litvak 2007a,b). Thus winter flounder are an ideal model species to examine sperm and egg interactions in the laboratory.

Here, we conducted a laboratory experiment to determine the optimal number of spermatozoa required to fertilize winter flounder eggs in a controlled setting. We also explore how long spermatozoa are viable and eggs are receptive after exposure to seawater. The hope is that this work will shed light on the reproductive ecology of this species.

MATERIALS AND METHODS

Broodstock husbandry and gamete collection

Adult winter flounder were obtained in March 2006 from the Western Passage of Passamaquoddy Bay, New Brunswick, Canada, using bottom trawl. Mean (± SD) total length of the broodstock was 26.7 ± 4.8 cm. After capture and transport, broodstock were held at the University of New Brunswick, Saint John, in a 2700 l closed recirculation system. Temperature and salinity ranged from 2 to 3°C, and 27 to 30 ppt, respectively. Dissolved oxygen remained above 10.1 mg l^{-1}. Broodstock were maintained under ambient photoperiod regime and were not fed while in captivity (e.g. Litvak 1999, Butts & Litvak 2007a,b).

Sperm cells were collected from 33 randomly selected adult male winter flounder. Sperm samples were obtained by applying light pressure on the abdomen and collecting semen in 3 ml syringes. To avoid seawater and urine contamination, the initial male ejaculate was discarded and the external urogenital pore was wiped dry with a paper towel. After stripping, syringes were held in a glass beaker placed in a cooler filled with crushed ice until used for experimentation. Spermatozoa motility was evaluated by placing 1 µl of spermatozoa on a microscope

slide, activated with 40 µl of seawater, and covered with a coverslip. Motility was estimated at 10 s post-activation using an arbitrary scale of 6 scores ranging from 0 to 5 where 0 = no motility, 1 = 1 to 19%, 2 = 20 to 39%, 3 = 40 to 59%, 4 = 60 to 79%, 5 = 80 to 100% (Suquet et al. 1998). Three replicate activations were completed for each male. For all activations, males had motility scores of 5.

Female broodstock were checked daily for signs of ovulation. Once the female's egg pore had swelled outside the body cavity, eggs were stripped by applying light pressure to the abdomen, and expelled eggs were collected into dry 100 ml beakers. Egg quality was immediately assessed after collection; eggs from females that were not clear and spherical were not used for experimentation.

All instruments and seawater used for fertilization were kept at 7 to 8°C. We conducted all fertilization trials and incubated the eggs inside a temperature-controlled room at 7 to 8°C prior to determination of fertilization success.

Expt 1: Determination of spermatozoa to egg ratio

Eggs from each of 5 females were crossed with the spermatozoa from 3 males. Different males (n = 3) were used for each female, resulting in a total of 15 parental half-sibling families. Seawater (28 ppt) contained 13 mg l^{-1} of penicillin G, and 13 mg l^{-1} of streptomycin sulfate (Butts & Litvak 2007a,b). After collection, eggs were placed into 100 × 15 mm Petri dishes (450 eggs per Petri dish, which is equal to ~0.1 ml; authors' unpubl. data), using a 1.0 ml syringe. Based on the experimental design we did not have enough time to determine the actual spermatozoa density for each male prior to experimentation (determined at a later stage; see below). Therefore, we used the value from Harmin & Crim (1993) of 1.64×10^{10} spermatozoa ml^{-1} to initially set the 6 spermatozoa to egg ratios we tested. We attempted to develop a range of spermatozoa to egg ratios on a logarithmic scale from 100 to 10 million spermatozoa per egg. Three replicate crosses were completed for each spermatozoa to egg ratio. Spermatozoa were added directly to the eggs in each dish using a dry fertilization approach. The spermatozoa-egg solution was gently swirled to homogenize the mixture and then 40 ml of seawater was added to activate the gametes. Using this fertilization technique will allow the eggs to stick to the bottom of the Petri dish in a mono-layer. Egg and spermatozoa contact time was set at approximately 5 min. After this period, gametes were rinsed with 20 ml of seawater, and then Petri dishes were refilled with 40 ml and left to incubate until being examined for fertilization success. Fertilization success was examined on a mean (±SEM) of 43.9 ± 0.74 eggs per Petri dish.

In order to determine the actual spermatozoa density per male we cryopreserved the fresh sperm from each male in liquid nitrogen (following Rideout et al. 2003) to be later analyzed. Frozen-thawed semen from each male was later counted under a compound microscope (Olympus BX-40) at 400× magnification using an improved Neubauer haemocytometer. Milt from each male was first diluted 500-fold in a sucrose-based diluent (125 mM Sucrose, 100 mM $KHCO_3$, 6.5 mM reduced glutathione 98%; pH 8.1) that does not activate sperm (Rideout et al. 2003). Milt-diluent samples were mixed on a vortex mixer in order to obtain a homogenous mixture. Triplicate dilutions were made for each male sample. Three counts of 5 squares (1 mm^2) were inspected for each dilution. The mean of the 3 counts for each dilution was calculated, and then the mean of these 2 values was used to determine the actual spermatozoa density ml^{-1} per male.

Expt 2: Spermatozoa longevity

Eggs from each of 3 females were crossed with the spermatozoa from 3 males producing 3 half-sibling families per female, and resulting in a total of 9 parental half-sibling families. Milt (1 ml) was diluted in 219 ml of seawater and mixed for 15 s. The next 20 ml of this solution was added to 450 eggs in a Petri dish (100 × 15 mm) after 30, 60, 120 and 240 s post-sperm activation. After ~5 min of gamete contact, excess spermatozoa were rinsed with 20 ml of seawater, and the Petri dishes were refilled with 40 ml of seawater and left to incubate. Fertilization success was examined on a mean (±SEM) of 18.5 ± 0.9 eggs per dish.

Expt 3: Egg longevity

Eggs from each of 3 females were crossed with the spermatozoa from 3 males producing 3 half-sibling families per female, resulting in a total of 9 parental half-sibling families. To perform experimental crosses, we first added 450 eggs to a dry petri dish (100 × 15 mm). Next, 10 ml of seawater was added to the eggs in each dish. The eggs remained in seawater for 30, 60, 120, 240, 480, 960, 1920, 3840 and 7680 s before semen was added for fertilization (according

to Expt 2). After ~5 min gamete contact, excess spermatozoa were rinsed with 20 ml of seawater, and sequentially refilled with 40 ml of seawater and left to incubate. Fertilization success was examined on mean (±SEM) of 58.7 ± 1.3 eggs per Petri dish.

Expts 1 to 3: Embryo incubation and fertilization success

Every second day, 75% of the sterilized seawater in each Petri dish was replaced. At 5 to 6 d post fertilization, egg and/or embryos were randomly sampled from each parental treatment combination and observed under low light using a dissecting microscope (Wild Heerbrugg; at 40× magnification) in order to determine fertilization success. Fertilization success was calculated as the percent fertilized eggs. Embryos that had developed to the late epiboly or early somite stage were considered fertilized.

Statistical analysis

All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute 2003). Residuals were tested for normality (Shapiro-Wilks test; PROC UNIVARIATE; SAS Institute 2003) and homogeneity of variance (plot of residuals vs. predicted values; PROC GPLOT; SAS Institute 2003). Fertilization success was arcsin square-root transformed (Zar 1996). Alpha was set at 0.05 for main effects and interactions.

Expt 1: Determination of spermatozoa to egg ratio

Segmented linear regression analysis was used to analyze the data based on the adjusted spermatozoa cell densities per male, and a breakpoint (or threshold) was calculated (SAS PROC NLIN; SAS Institute 2003). Segmented linear regression is an iterative model that, if valid, will converge to solve for a breakpoint between 2 linear relationships: one slope and one asymptote. In our study this breakpoint reflects the number of spermatozoa after which adding more spermatozoa to the eggs will not significantly increase the percent fertilization (following Robbins et al. 2006). Breakpoints were generated for each male-female combination. The parameters for the segmented regression were defined as R, the breakpoint *x*-value, and L, the asymptote on the *y*-axis at R (Robbins et al. 2006).

Expts 2 and 3: Spermatozoa and egg longevity

Fertilization success (Y) was analyzed using a mixed-model nested factorial ANOVA (PROC MIXED; SAS Institute 2003):

$$Y_{ikpn} = \mu + A_i + B(A)_{k(i)} + C_p + AC_{ip} + BC(A)_{kp(i)} + \varepsilon_{n(ikp)}$$

where μ is the true mean; A_i is the female effect (where i = 1–3); $B(A)_{k(i)}$ is the male effect (where k = 1–4) nested within female; C_p is the effect of gamete longevity (where p = 1–4 for sperm longevity and 1–8 for egg longevity); AC_{ip} is the female × gamete contact interaction; $BC(A)_{kp(i)}$ is the male × gamete contact nested within female interaction; and $\varepsilon_{n(ikp)}$ is the residual error. Female and male effects were considered random, while gamete longevity was considered fixed. Denominator degrees of freedom for all *F*-tests were approximated using the Satterthwaite procedure (Satterthwaite 1946). *A posteriori* analyses performed on fixed effects were constructed using Tukey's multiple comparisons procedure. Note that in SAS PROC MIXED models, *F*-ratios and p-values are only generated for fixed effects (Littell et al. 1996, SAS Institute 2003).

RESULTS

Expt 1: Determination of spermatozoa to egg ratio

Spermatozoa densities for the males ranged from 8.10×10^9 to 17.7×10^9 sperm cells ml^{-1} (Table 1). All segmented regressions models successfully converged except for Male 9. Models were significant for each of the males ($F_{2,27} \geq 26.1$; $p < 0.0001$). Mean and confidence limits for L and R were calculated for each male (Fig. 1; Table 2). On average 34 038 sperm cells per egg were required to fertilize 81.3% of the eggs.

Table 1. *Pseudopleuronectes americanus.* Actual spermatozoa densities and the mean for the 15 winter flounder males used in Expt 1

Male	Spermatozoa ml^{-1}	Male	Spermatozoa ml^{-1}
1	8.5×10^9	9	14.1×10^9
2	12.4×10^9	10	11.4×10^9
3	17.7×10^9	11	10.4×10^9
4	9.6×10^9	12	9.5×10^9
5	11.5×10^9	13	8.1×10^9
6	10.1×10^9	14	10.1×10^9
7	8.8×10^9	15	10.9×10^9
8	14.6×10^9	Mean	11.2×10^9

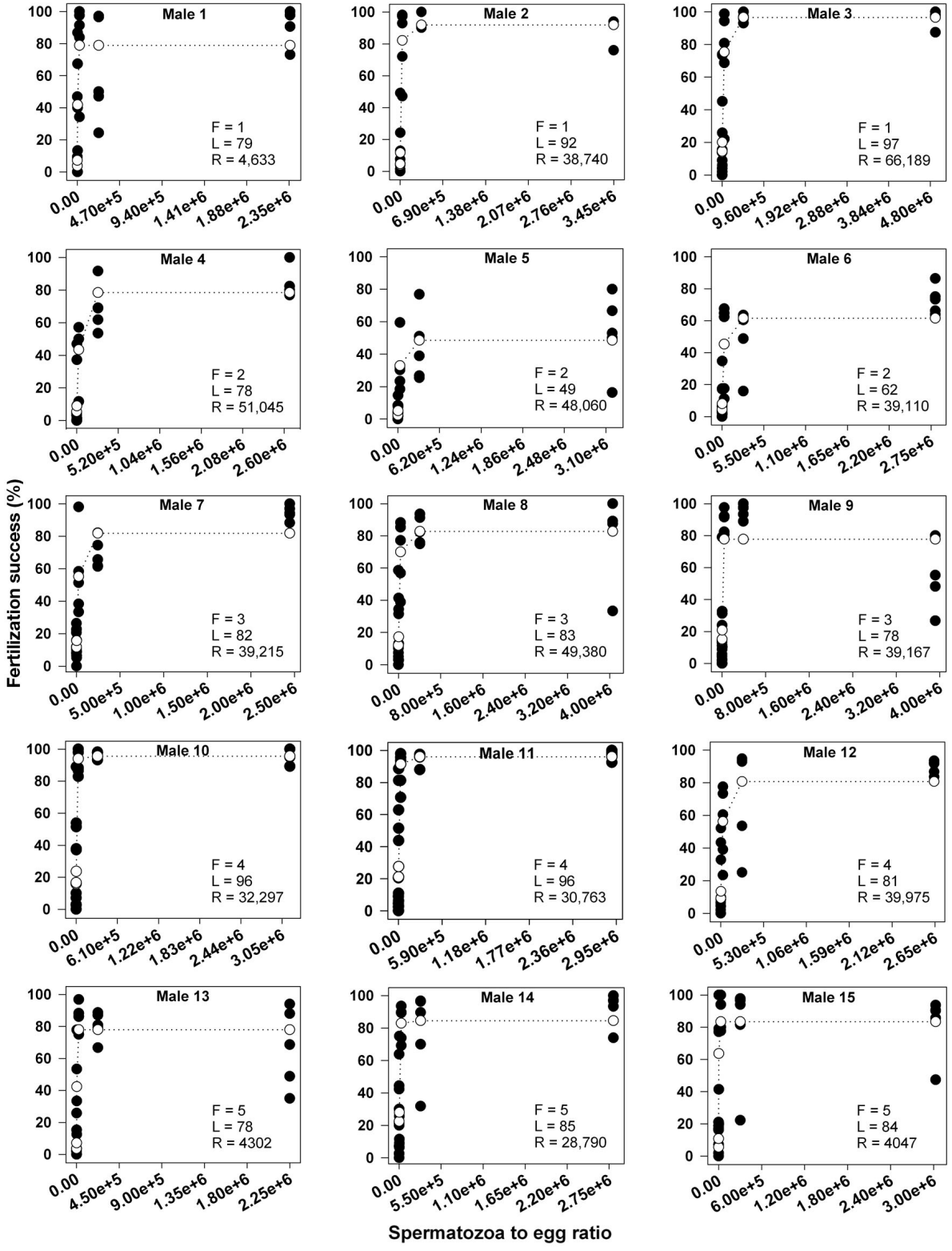

Fig. 1. *Pseudopleuronectes americanus.* In Expt 1, segmented linear regression analysis was used to analyze the winter flounder data and a breakpoint was calculated for each male-female combination. This breakpoint reflects the number of spermatozoa after which adding more spermatozoa to the eggs will not significantly increase the percent fertilization. F = female number, R = breakpoint *x*-value (spermatozoa to egg ratio), L = the asymptote on the *y*-axis at R (% of eggs fertilized), open circles = predicted values, closed circles = observed values

Table 2. *Pseudopleuronectes americanus.* In Expt 1, segmented linear regression analysis was used to analyze the data, based on the adjusted sperm cell densities per winter flounder male. A breakpoint was calculated for each male-female combination. Breakpoints reflect the number of spermatozoa after which adding more spermatozoa to the eggs did not significantly increase the percent fertilization. The parameters for the segmented regression were defined as R, the breakpoint *x*-value (number of sperm cells), and L (expressed as % fertilization), the asymptote on the *y*-axis at R (Robbins et al. 2006). Confidence limits (lower and upper) for R and L were calculated for each male-female combination and mean values presented

Female	Male	L	R	L_{lower}	L_{upper}	R_{lower}	R_{upper}
1	1	78.9	4633.0	65.7	92.1	1520.1	7745.9
1	2	91.9	38740.0	81.6	102.2	31159.4	46320.9
1	3	96.6	66188.7	82.9	110.3	42260.3	90117.2
2	4	78.4	51045.0	68.5	88.4	28062.2	74028.4
2	5	48.5	48060.4	39.2	57.7	25002.3	71118.5
2	6	61.5	39109.7	50.8	72.3	22729.1	55490.3
3	7	81.8	39214.6	72.4	91.2	25852.6	52576.6
3	8	82.8	49380.4	70.7	94.9	32552.7	66208.2
3	9[a]	77.7	39166.7	65.7	89.6	–115340.0	193673.0
4	10	95.6	32297.0	83.9	107.2	24035.4	40559.8
4	11	96.0	30762.7	82.1	110.0	20491.9	41033.5
4	12	80.7	39974.6	68.0	93.4	22918.2	57031.0
5	13	78.0	4302.0	69.4	86.5	2467.8	6136.8
5	14	84.6	28789.6	70.9	98.3	17745.6	39833.7
5	15	83.5	4047.0	72.7	94.2	2612.8	5480.6
Mean		81.3	34038.9	69.9	92.8	21386.5	46691.5

[a]The segmented regression model for Male 9 did not successfully converge, therefore Male 9 values were not used to calculate the mean for each parameter estimate

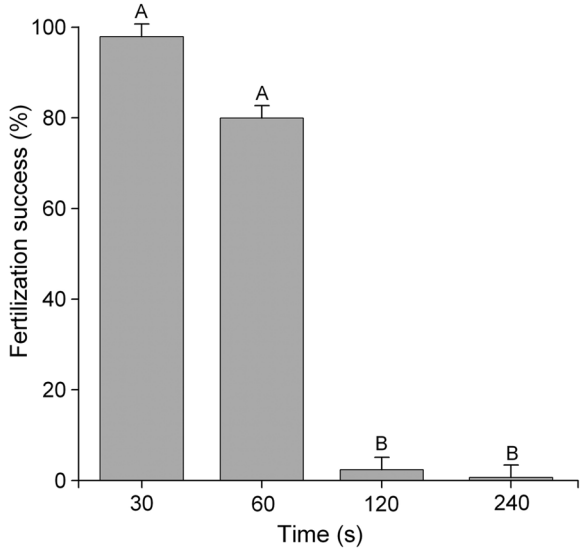

Fig. 2. *Pseudopleuronectes americanus.* Length of time spermatozoa (30–240 s post-activation) are capable of fertilizing eggs after exposure to seawater. Error bars represent least square means standard error (PROC MIXED; SAS Institute 2003). Bars with different letters are significantly different ($p < 0.05$, least square means, ANOVA)

Expt 2: Spermatozoa longevity

Duration after spermatozoa activation had a significant effect on proportion of eggs fertilized ($F_{3,6.69} = 338.38$; $p < 0.0001$; Fig. 2). At 30 s post-spermatozoa activation, 98% of the eggs were fertilized on average. At 120 s post-spermatozoa activation, we observed a significant decrease in fertilization success.

Expt 3: Egg longevity

Duration after egg exposure to seawater had a significant effect on proportion of eggs fertilized ($F_{8,16} = 19.89$; $p < 0.0001$; Fig. 3). Between 30 and 1920 s after exposure to seawater, the percentage of eggs fertilized ranged from 61 to 90%. A significant decrease to 11% occurred at 3840 s after egg exposure to seawater (Fig. 3).

DISCUSSION

Spermatozoa to egg ratios can inform us about the reproductive ecology of fishes. To date including the present study, we currently know spermatozoa to egg ratios for only 15 of the ~28 000 species of fishes (Table 3). We found, as would be expected, that increasing spermatozoa cell density increased fertilization success. However, the position of the breakpoints for the segmented regressions were highly variable between males, suggesting the fertilization potential is specific to the individual male; this is a result of a male only contributing sperm to reproduction. When considering our fish's spermatozoa traits, all males had high (≥80%) motility. Butts & Litvak (2007a) examined the contribution of parental effects on reproductive success and found that the paternity significantly influenced fertilization in winter flounder. Therefore, further work is required to establish a linkage between spermatozoa traits (i.e. adenosine-5′-triphosphate concentration, morphology, seminal plasma biochemistry; Lahnsteiner et al. 1998, Asturiano et al. 2005, Alavi et al. 2011, Butts et al. 2011) and fertilization ability for this species. Such information would be an important early indicator for evaluation of a male's reproductive potential (Trippel 2003).

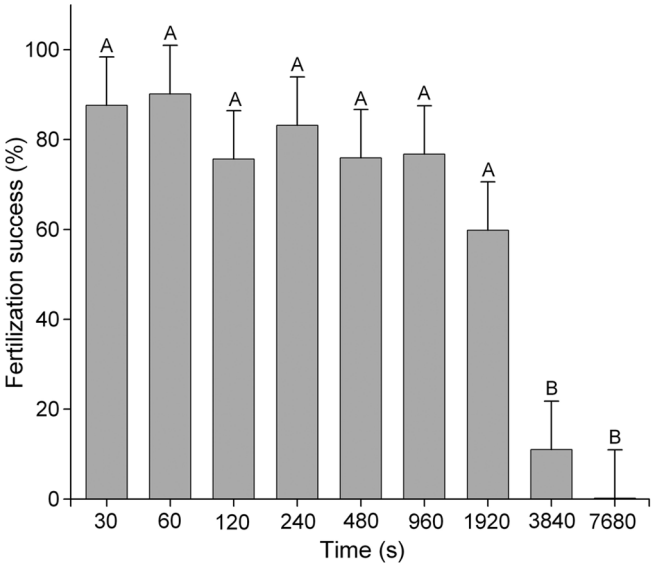

Fig. 3. *Pseudopleuronectes americanus.* Length of time eggs (30–7680 s post-activation) are receptive to fertilization after exposure to seawater. Error bars represent least square means standard error (PROC MIXED; SAS Institute 2003). Bars with different letters are significantly different ($p < 0.05$, least square means, ANOVA)

The number of spermatozoa needed to fertilize one ovum in fish is relatively high and species dependent, and even within the same species different optimal spermatozoa to egg ratios have been reported by different studies (Table 3). For example, rainbow trout *Oncorhynchus mykiss* was found to have an optimal spermatozoa to egg ratio of 75 000 (Erdahl & Graham 1987) and 300 000 (Billard 1975). This intraspecies variability may be an artifact of different fertilization environments (i.e. amount of water used to activate the gametes; Table 3) or may be attributed to gamete quality at the time of stripping. Nevertheless, such high numbers of spermatozoa are necessary for fertilization since each egg has a unique point(s) of penetration, the micropyle, which the spermatozoa have little chance of reaching when their concentration is low or when their motility, velocity, or longevity (among others) is reduced. In the present study, the lower and upper confidence limits of 21386 to 46 691 winter flounder spermatozoa per egg are required to fertilize on average 81.3% of the eggs, which is in the middle of the range of spermatozoa to egg ratios currently found in the literature (Table 3).

Spermatozoa to egg ratio results from the present study are useful for laboratory work on winter flounder as well as in modelling what may be occurring in nature. This approach can provide insight into spawning behavior and ecology in winter flounder. For example, using our data, and that from the literature — (1) winter flounder females are single batch spawners producing between 192 240 and 3.33 million eggs per season (Scott & Scott 1988, Buckley et al. 1991); and (2) males, which recruit their spermatogonia in the fall, have a finite supply of spermatozoa — we can generate a simple model to inspect the spawning ecology of winter flounder. Since males produce

Table 3. The optimal number of spermatozoa needed to fertilize an ovum in fish. Spermatozoa:egg ratio reflects the number of spermatozoa after which adding more spermatozoa to the eggs will not significantly increase fertilization success; AM = activation medium; ^{DF} = dry fertilization; ^{WF} = wet fertilization; nd: no data

Fish species	Spermatozoa:egg ratio	Volume of AM	Reference
African catfish *Clarias gariepinus*	15000	10 ml	Rurangwa et al. (1998)
Asian catfish *Clarias macrocephalus*	4000	0.5 ml g egg^{-1}	Tambasen-Cheong et al. (1995)
Atlantic cod *Gadus morhua*	100000	40 ml	Butts et al. (2009)
Brown trout *Salmo trutta*	43000	nd	Erdahl & Graham (1987)
Common carp *Cyprinus carpio*	236720	5 ml	Linhart et al. (2003)
European catfish *Silurus glanis*	800–80000	6 ml	Linhart et al. (2004)
Herring *Clupea harengus*	9600000	nd	Rosenthal et al. (1988)
Northern pike *Esox Lucius*	26000	nd	Erdahl & Graham (1987)
Pacu *Piaractus mesopotamicus*	7000	15–60 ml	Sanches et al. (2011)
Rainbow trout *Oncorhynchus mykiss*	300000	nd	Billard (1975)
	75000	nd	Erdahl & Graham (1987)
Sea lamprey *Petromyzon marinus*	50000	10 ml	Ciereszko et al. (2000)
Turbot *Scophthalmus maximus*	6000	1–19 ml	Suquet et al. (1995)
	>9000^{DF}/3000–4000^{WF}	100 ml	Chereguini et al. (1999)
Walleye *Sander vitreus*	25000	nd	Rinchard et al. (2005)
	500000	400 ml	Casselman et al. (2006)
Winter flounder *Pseudopleuronectes americanus*	34038	40 ml	This study
Wolffish *Anarhichas lupus*	200000	nd	Moksness & Pavlov (1996)

on average 2.2 ml of milt per season, at an average spermatozoa density of 1.64×10^{10} per ml, the total number of spermatozoa produced per season is 3.6×10^{10} (Harmin & Crim 1993, this study). Therefore, the ratio of spermatozoa to eggs for a less fecund female (192 240 eggs) is 187 682:1; for a highly fecund female (3.33 million eggs) it is 10 835:1. Clearly, if an average male encountered a lower fecund female, he would be able to fertilize all of her eggs as the number of spermatozoa per egg ratio is less than the optimal observed for our fish. However, if an average male were to encounter a large and highly fecund female, he would not be able to fertilize all her eggs, as the spermatozoa per egg ratio is below our confidence limits. One caveat here is that fertilization conditions in the laboratory are perfect; i.e. no currents advecting away the spermatozoa as well as spermatozoa and eggs placed in close proximity for a long duration. Therefore, we would expect that spermatozoa contact time and density in the wild should be much less, suggesting that it might take more than 1 male to fertilize the eggs of 1 gravid female. Overall, these results suggest that winter flounder may be promiscuous, and a skewed sex ratio towards males may be advantageous in the wild to maintain recruitment.

Most research on spermatozoa longevity has focused on freshwater species, for which spermatozoa longevity persists for 1 to 2 min while marine species can remain motile up to 2 h (reviewed by Suquet et al. 1994). Winter flounder males do not seem to have spermatozoa that remain viable as long as other marine species. However, we found that winter flounder eggs can be fertilized for up to 32 min after the female has released eggs into seawater. These 2 points, in addition to the spermatozoa to egg ratio results, also suggest promiscuous mating strategies for this species. Increased egg receptivity will allow a female a greater chance to have her eggs fertilized by more than 1 male. Additionally, if males ration their spermatozoa and fertilize more than 1 female's eggs, then this too suggests multiple partners for reproduction. Stoner et al. (1999) examined mating behavior of winter flounder in tanks and found that spawning most often involved multiple males. Our results, in combination with that of Stoner et al. (1999), suggest multiple mates for females.

Studies on optimal spermatozoa to egg ratios and gamete interactions are also extremely important for routine production of winter flounder for aquaculture and conservation efforts. Ultimately, both these efforts can be enhanced with the use of cryogenic technology, as the cryopreservation process has been shown to have no effect on a sperm cell's ability to fertilize an egg for this species (Rideout et al. 2003). Once frozen, sperm cells from different stocks and lineages become a 'priceless commodity'; i.e. frozen-thawed cells can be used to perform what is called 'genetic rescue' if it is needed. Therefore, using our results we can now determine how many eggs each cryogenic spermatozoa straw can fertilize for routine production. By taking the average number of spermatozoa from the 15 males used in Expt 1, which equals 1.12×10^{10} sperm cells ml^{-1}, each 0.25 ml cryogenic sperm straw (containing 1 part sperm to 3 parts extender) contains 0.0625 ml or 7.0×10^{8} sperm cells. Using the mean ratio of 34 038 spermatozoa to egg, each cryopreserved straw should therefore be able to fertilize ~20 565 eggs.

In conclusion, the information provided here is pertinent to fisheries ecologists and also has implications for selective breeding programs and aquaculture production of marine flatfishes. Additionally, we show that studying gamete biology can provide an opportunity to learn much about a species reproductive ecology in a very short period of time.

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