

Relationship between fertilization success and the number of milt donors in rainbow smelt *Osmerus mordax* (Mitchell): implications for population growth rates

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A series of artificial fertilizations using rainbow smelt *Osmerus mordax* were conducted, each designed to assess the fertilization success of a female's eggs with milt from one to five males. Mean egg fertilization success increased and variance in fertilization success decreased when more males contributed to a constant volume of milt. This may indicate the presence of an Allee effect. The results suggest that potential changes in egg fertilization success due to a decrease in the number of males, in addition to changes in egg production due to a decrease in the number of females, can affect estimates of maximum sustainable harvest rates, and recovery of depleted populations.

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INTRODUCTION

In fish species with external fertilization, *e.g.* Atlantic cod *Gadus morhua* L. (Brawn, 1961; Hutchings *et al.*, 1999), Atlantic salmon *Salmo salar* L. (Jones, 1959) and bluegill sunfish *Lepomis macrochirus* Rafinesque (Gross & Charnov, 1980), spawning often involves several males releasing milt as a single female releases eggs. Mating systems that involve multiple males include broadcast spawners and alternative male mating strategies (Shuster & Wade, 2003). Variance in male reproductive success is reduced in these types of mating systems (Hutchings & Myers, 1988; Jones & Hutchings, 2001), and it is presumed that females benefit by increasing genetic variation among their offspring (Jennions & Petrie, 2000). Females may also benefit by increasing egg fertilization success (Rowe *et al.*, 2004). There is a long-standing assumption that sperm is not limiting in fish populations and the number of males is often ignored when determining larval production. Differences among males in milt quality, however, can influence fertilization success (Burness *et al.*, 2004). This may be another

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explanation for multiple male matings and indicates that the number of mature males in a population may be a more important determinant in recruitment than previously thought.

Globally, many fish species have experienced dramatic declines in population abundance (Hutchings, 2000; Myers & Worm, 2003). Despite the expectation that density-dependent mechanisms lead to high recruitment and growth rates at low population sizes, populations that have undergone serious declines are slow, if ever, to recover (Hutchings & Reynolds, 2004). An Allee effect (Allee, 1931; also known in the fisheries literature as depensation) results in reduced per capita recruitment at low population sizes (Levitan *et al.*, 1992; Shelton & Healey, 1999; Frank & Brickman, 2000; Liermann & Hilborn, 2001), and has been invoked as a mechanism for the lack of recovery.

Maximum sustainable harvest rates are a type of fisheries management strategy, and can be estimated from life-history data (Shuter *et al.*, 1998; Purchase *et al.*, 2005). When smaller population sizes reduce total egg production, future sustainable harvests will decrease (assuming no density-dependent increases in survival or growth rates). In addition, a secondary factor influencing population growth may be egg fertilization success. For example, when fewer captive male Atlantic cod were involved in fertilizing a batch of eggs, the fertilization success decreased and the variance in the proportion of eggs fertilized increased (Rowe *et al.*, 2004). Reduced fertilization success may result from reduced opportunities for mate choice by females (Rowe & Hutchings, 2003), including the importance of size matching between males and females (Rakitin *et al.*, 2001), or genetic incompatibilities (Zeh & Zeh, 1996, 1997; Wirtz, 1997). Consequently, when populations and the number of males involved in single spawning events decline, resulting in reduced fertilization success (Rowe *et al.*, 2004), Allee effects may further exacerbate the effects of low population size on population recruitment.

To address this issue, experiments were conducted with rainbow smelt *Osmerus mordax* (Mitchell) to separate pre- from post-gamete contact as mechanisms influencing fertilization success. The experiments were designed to eliminate any effects of mating prior to gamete mixing. Rainbow smelt spawn in streams, where adhesive eggs are discharged directly into the water and are given no parental care (Scott & Crossman, 1973). Females are batch spawners, and a number of males may be present during each spawning event, which probably leads to multiple paternity. The main objectives of the present study were to test the hypotheses that fertilization success is higher and less variable when more males contribute sperm to a given volume of milt. Also investigated was whether male size influenced fertilization success, independently of the amount of milt produced. Additionally, the sensitivity of maximum sustainable harvest rates to differences in fertilization success was assessed.

MATERIALS AND METHODS

COLLECTION OF FISH

The study population of rainbow smelt reproduces in Steven's Brook, Nova Scotia, Canada (44°45' N; 63°05' W). The brook is <2 m in width and 15 cm in depth, allowing

rainbow smelt to be easily captured when in the system. Anadromous rainbow smelt enter the brook to spawn on a rising tide at night. Fish were collected from the brook at 2030 hours on 21 June 2005 (trial 1), at 2230 hours on 23 June 2005 (trial 2) and at 0145 hours on 29 June 2005 (trial 3), consistent with tidal cycles. The fish from which gametes were taken were individually frozen for later sampling of fork length (L_F ; mm) and body mass (M ; g), (Table I).

COLLECTION OF GAMETES

The fertilization experiments required gametes from five males and two females for each trial. Gametes from eight males and four females, however, were taken to ensure samples of good quality. Fish that easily released eggs or milt with gentle pressure were selected. Preliminary experiments indicated that a batch of eggs had to be 'runny' enough not to form peaks in a Petri dish in order to get >50% fertilization success.

Selected fish were killed by a blow to the head and the area surrounding the urogenital opening was dried using a paper towel prior to gamete extrusion. Two milt samples from each male were collected using 1.0 ml syringes, transferred to separately labelled 0.5 ml Eppendorf tubes, and placed directly in crushed ice. Eggs from a single female were squeezed into a small plastic bag, which was then inflated with air and placed on top of paper towels in a cooler containing ice. Samples containing urine or faeces were discarded. Later, gametes were removed from the cooler and gradually warmed to the fertilization temperature before mixing.

TABLE I. Fork length (L_F), body mass (M), male residual mass (M_R) and mean fertilization success of rainbow smelt used in the fertilization experiments

Trial	Sex	Identity	L_F (mm)	M (g)	M_R (unitless)	Mean fertilization success (%)
1	M	1	175	47.9	0.0323	0.8
	M	2	192	60.5	0.0159	59.2
	M	3	192	42.8	-0.1344	67.5
	M	4	168	42.1	0.0281	63.6
	M	5	155	34.9	0.0489	54.2
	F	A	206	62.9		
	F	B	175	40.9		
2	M	1	147	24.2	-0.0428	70.7
	M	2	134	18.6	-0.0395	4.3
	M	3	145	28.3	0.0426	1.0
	M	4	143	27.0	0.0398	46.3
	M	5	170	49.1	0.0798	56.9
	F	A	200	67.4		
	F	B	176	41.9		
3	M	1	171	39.1	-0.0265	61.0
	M	2	168	41.8	0.0249	56.8
	M	3	162	39.7	0.0488	68.8
	M	4	144	20.0	-0.0994	23.3
	M	5	147	25.6	-0.0184	0.0
	F	A	206	71.5		
	F	B	181	46.2		

ARTIFICIAL FERTILIZATIONS

During each trial, two females and five males were used (total = six females and 15 males). Fertilizations were conducted in standard Petri dishes, containing 0.05 ml of eggs from a given female (c. 100 eggs). There were 50 Petri dishes per female giving a total of 100 gamete mixtures for each trial. Each male was crossed with both females. The total design was repeated over three trials, giving a grand total of 300 fertilization procedures.

The design was a four-way blocked ANOVA (Fig. 1). The independent variables were the number of males used (d.f. = 4), female identity (d.f. = 1), the order in which the males were used (d.f. = 4, called combination) and the three trials where the experiment was repeated (blocking factor, d.f. = 2). The males were numbered 1–5 at random. Each primary treatment (the number of males) was replicated identically two times in five combinations (see Fig. 1). For example, in combination A, male number 1 was used in all treatments (two replicates for one male, two replicates for two males, two replicates for three males, two replicates for four males and two replicates for five males), male number 2 in treatments 2–5, male number 3 in treatments 3–5, male number 4 in treatments 4–5 and male number 5 only in treatment 5. For combination B, male number 2 was used in all treatments (two replicates for each of five groups), and so on (Fig. 1). The two identical procedural replicates were averaged for statistical analyses.

Prior to starting the fertilization procedure all milt samples were checked for sperm motility. Approximately 0.025 ml of dechlorinated water was added to a slide and a micropipette tip was used to add a very small amount of milt, which was immediately mixed. Sperm motility was assessed under a compound microscope at $\times 200$ magnification. Five males that demonstrated good (>50%) sperm motility were selected for each trial. The same volume of milt was added to each Petri dish of eggs; no adjustment was made for potential differences in spermatocrit. Thus, in the treatment with one male, five times more milt was added from a single individual than the treatment with five males. The total amount of milt added was 5% of the egg volume (0.0025 ml),

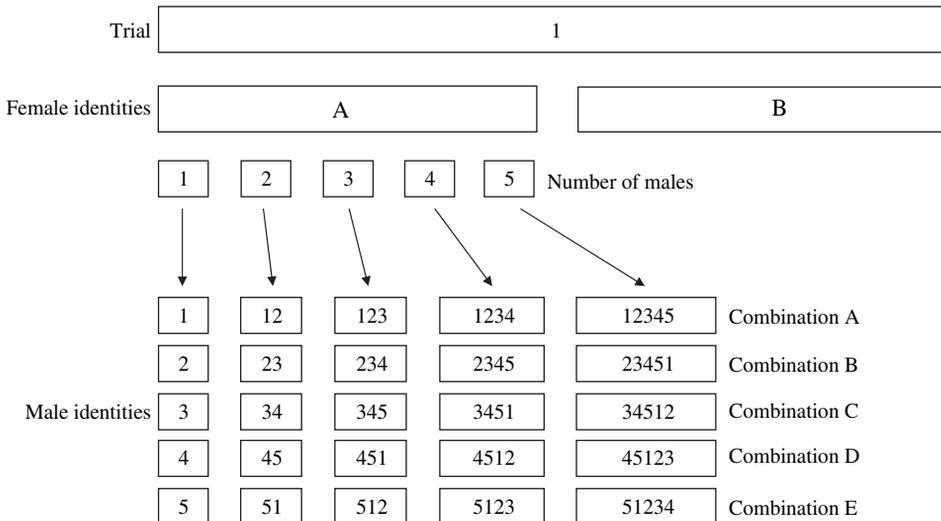


FIG. 1. Schematic of study design. During each trial (total of three trials) both females were crossed with the same milt mixtures. There were five levels to the main treatment (number of males) and five combinations for each level (various males used). The total amount of milt used was the same for every cross. Shown here is replicate 1 for female A in trial 1. There were two replicates for each cross, giving 100 crosses per trial.

which was 2.5% of the fertilization medium, thus ensuring there was enough milt to accurately pipette from each male.

Milt was added and mixed dry with eggs, after which 0.05 ml of dechlorinated tap water was added to activate sperm. After *c.* 5 min the bottoms of the Petri dishes were covered with water (prior microscopic examination indicated that sperm only swam for *c.* 5 s). The first replicates (50 Petri dishes) used one of the vials of sperm from each male, and were completed before any of the second replicate crosses were begun. It took *c.* 45 min to get the first replicate of all 50 fertilizations completed; 1 h passed before the procedure started for replicate 2 (using the same egg source and the same males, but different vials of milt).

All fertilizations and incubations were conducted at *c.* 20° C, which was near to that in the stream at the time fish were spawning (consistently measured at >18° C). Three hours after gamete mixing, Petri dishes were placed on a grid under a dissecting microscope to determine the proportion of eggs that had been fertilized, as indicated by observations of cellular division (dividing eggs reached at least the four cell stage in 3 h).

STATISTICAL ANALYSES

Per cent fertilization was arcsine square root transformed prior to statistical analyses (Zar, 1974). Trial, combination and female were set as random factors; the number of males was a fixed categorical factor. Given that trials were replicate blocks it was not used in any interaction term. All other interaction terms were first included in the full model and subsequently dropped if not significant. Significance level was set at 0.05 and assumptions of parametric statistics were checked by examining residuals. To assess the magnitude of the effect of different numbers of milt donors, the average fertilization success was taken between replicates, females, trials and combinations for the entire study, and line of best fit determined for the resulting five data points.

Residual (relative) body mass (M_R) of each male was determined from a $\log_{10} L_F$ and $\log_{10} M$ regression. Fertilization success among males was compared using the one male treatment. The model terms were block, female, male size (covariate) and the interaction term between male size and female. Three separate analyses used male L_F , M and M_R as the independent variables. A Bonferroni correction was applied giving a critical α of 0.0167.

ESTIMATION OF MAXIMUM SUSTAINABLE HARVEST RATE IN A HYPOTHETICAL POPULATION

Sensitivity of an estimated maximum sustainable harvest rate to variable fertilization success was assessed for a hypothetical rainbow smelt population. The procedure used was that reported in Purchase *et al.* (2005). The Malthusian parameter (r , per capita population growth rate) was calculated under two assumptions (no density-dependence and no anthropogenic mortality). Thus, under optimal conditions, the estimated r is the maximum possible (r_{\max}). Since rainbow smelt have a distinct reproductive season, an estimate for r_{\max} could be made using an age-structured Leslie matrix model (Caswell, 1989; Hayes, 2000): $1 = \sum_{x=1}^q l_x m_x e^{-rx}$, where l_x and m_x are age specific survival and birth rates respectively, and q is the life span.

The hypothetical rainbow smelt population had life-history characteristics typical for the species (Scott & Crossman, 1973; Froese & Pauly, 2005). These were growth rate (von Bertalanffy parameters, $L_\infty = 28.8$ cm, $k = 0.415$), a L_F and M relationship ($M = aL_F^b$, where $a = 0.0118$ and $b = 2.9030$), maturation age (2 years), relative fecundity (440 eggs g^{-1} of fish), life span (5 years), mortality to age 1 (no data available, set at 99.9%, the value does not affect the conclusions) and adult mortality [estimated using Hoenig's equation (Hoenig, 1983) and was equal to 86.9%]. Fecundity was

divided in half in order to obtain the female birth rate. These life-history parameters were used to determine r_{\max} using the solver function in Microsoft Excel™.

To obtain estimates of l_x the population started at age 0 year at an arbitrary size. The mortality rate to age 1 year was applied to determine the proportion of individuals remaining after 1 year. The adult mortality rate was used in each subsequent year to obtain the proportion of individuals remaining in each age class. Estimates of m_x were obtained for each mature age class, which were ages 2–5 years. At each age class, the mean mass of an individual was obtained using the von Bertalanffy equation and the rainbow smelt specific L_F and M conversion. Mean M was multiplied by relative fecundity to obtain mean fecundity at a given age, which was divided in half.

The index of fitness (r_{\max}) was used to estimate sustainability. For each population, an external adult mortality (fishing) was applied to the model and increased until the calculated r was reduced to zero. The level of fishing mortality that reduced r to zero is the maximum sustainable fishing rate; any rate exceeding this value will drive the population to extinction, assuming no density-dependent changes in growth, survival or reproduction. The purpose was not to estimate what the maximum sustainable fishing rate actually was for this population, but to compare relative differences in the estimate based on differences in fertilization success. To do this the above procedure was repeated using 100% egg fertilization (no extra mortality), and that predicted by the five experimental treatments.

RESULTS

The artificial fertilization procedure resulted in a median and mean fertilization success of 65.8 and 58.1% respectively. The fertilization success for each of the 300 gamete mixtures is presented in Fig. 2. For reasons unexplained, in trial 1 the eggs belonging to female A became very viscous during the hour wait between the first and second replicate. This may have caused the substantial drop in mean fertilization success for the second replicate. Because it occurred for all treatments, however, it added error, but no bias, to the overall design (thus reducing statistical power).

None of the interaction terms were significant and were subsequently removed from analyses. Fertilization success increased significantly with the number of males contributing milt (ANOVA, d.f. = 4,138, $P < 0.001$; Figs 2 and 3). Tukey *post-hoc* tests indicated that fertilization success for the one male treatment differed from all others, but there were no significant differences among the other treatments. Predicted fertilization successes for the different number of males were 1 = 41.3%, 2 = 56.7%, 3 = 62.3%, 4 = 64.4% and 5 = 65.2%. An anticipated result was that fertilization success varied significantly among trials (ANOVA, d.f. = 2,138, $P < 0.05$). The combination (males used in each cross) was not significant (ANOVA, d.f. = 4,138, $P > 0.05$). As expected, within each trial, the fertilization success was significantly different between females (ANOVA, d.f. = 1,138, $P < 0.01$; Fig. 2).

The increase in fertilization success with the number of males appeared to be associated with a decrease in the variability in fertilization success among combinations (the five different data points for a given number of males in Fig. 2). To analyse this trend the coefficient of variation (CV) was used as a metric for variability, as this controls for differences in the mean fertilization success. The CV decreased significantly with the number of males contributing milt (ANOVA, d.f. = 4,22, $P < 0.001$; Fig. 4). Tukey *post-hoc* tests indicated the one male treatment was significantly different from all others, the two male treatment different

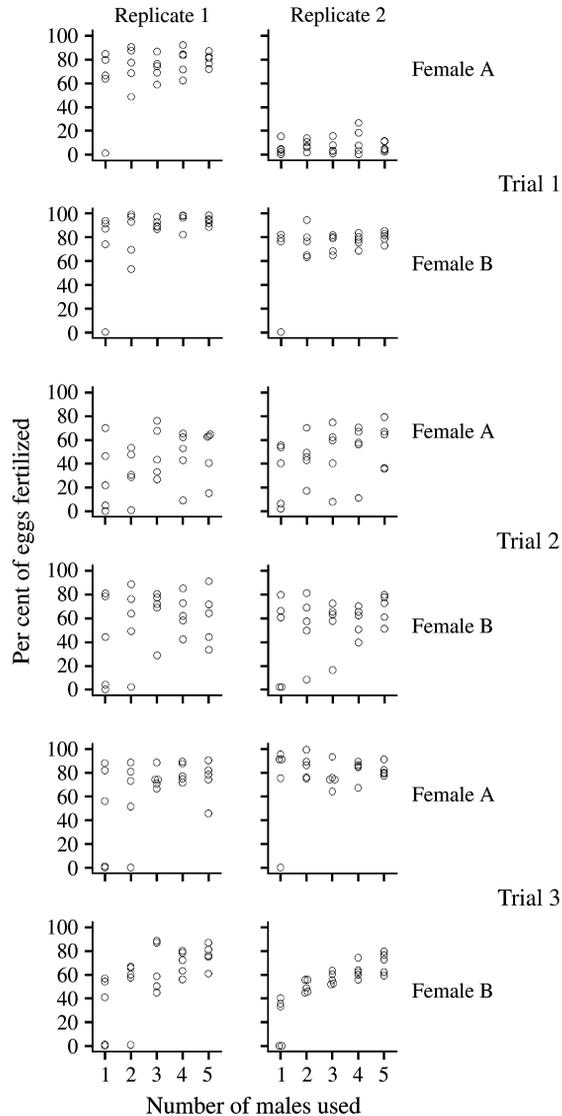


FIG. 2. Artificial fertilization success of rainbow smelt eggs. Sperm motility of males was checked before the experiment started. The same volume of milt was used in each treatment. All in replicate 1 were done before any in replicate 2. Some symbols have been slightly offset on the *x*-axis to prevent complete overlap.

from four and five males, and no differences among the other treatments. Predicted CVs in fertilization success for the different number of males are 1 = 74.2%, 2 = 31.9%, 3 = 19.5%, 4 = 13.7% and 5 = 10.4%. The CV in fertilization success among combinations varied by trial (ANOVA, d.f. = 2,22, $P < 0.001$) but not between females within a trial (ANOVA, d.f. = 1,22, $P > 0.05$).

Male body size and mean fertilization success of individual males in the single male treatment are presented in Table I. None of the interaction terms

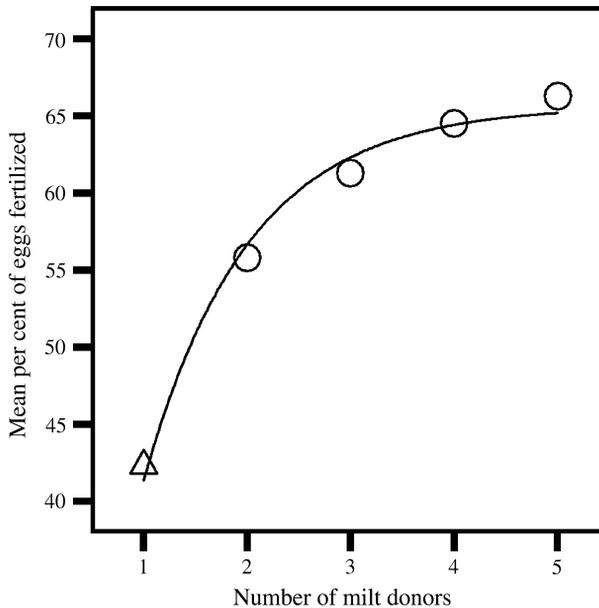


FIG. 3. Mean fertilization success of rainbow smelt eggs as a function of the number of milt donors. Each datum is the mean among females, combinations and trials. The curve was fitted by $y = 65.67(1 - e^{-0.993x})$ ($r^2 = 0.990$).

between male size and female size were significant, and were subsequently removed from analysis. Using Bonferroni correction ($\alpha = 0.0167$), male body size did not influence fertilization success for any of the variables: L_F (ANCOVA, d.f. = 1,25, $P > 0.0167$), M (ANCOVA, d.f. = 1,25, $P > 0.0167$) and M_R (ANCOVA, d.f. = 1,25, $P > 0.0167$).

Estimated r_{\max} of the hypothetical rainbow smelt population ranged from 2.22 with 100% fertilization success to 1.34 with 41.3% fertilization success (mean when a single male was involved in egg fertilization). The estimated maximum sustainable harvest rates were much lower than that predicted by 100% fertilization success (Fig. 5), and is predicted to decline when fewer males spawn with a female, the most substantial reduction (22.4%) being when a single male contributes milt instead of two (Fig. 5).

DISCUSSION

In many fish species multiple males may be involved in spawning with a single female as she releases a batch of eggs. While this is beneficial for males because they obtain some fitness, the current experiment with rainbow smelt indicates that polyandry may be a good strategy for females too because of predicted increased fertilization success and reduced variability in fertilization success among batches of eggs. At the population level, fertilization success can affect the intrinsic rate of increase, and ideally should be incorporated into models of maximum sustainable harvest rates.

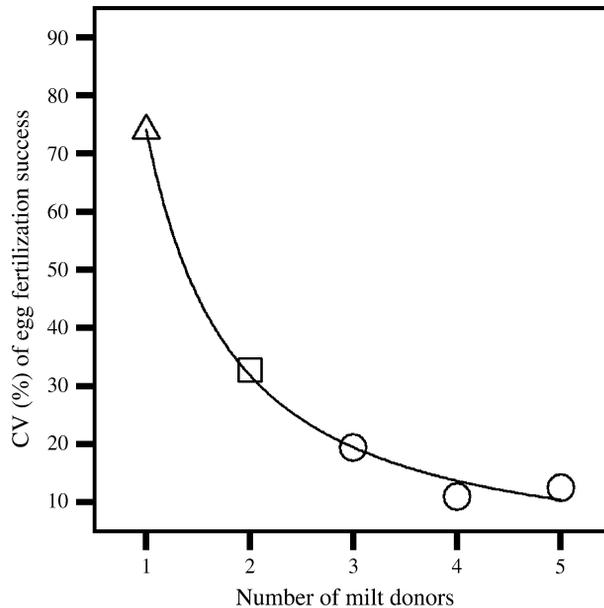


FIG. 4. Coefficient of variation (CV) in the fertilization success of rainbow smelt eggs among different combinations of males as a function of the number of milt donors. Each datum is the mean among females and trials. The curve was fitted by $y = 74.15x^{-1.218}$ ($r^2 = 0.995$).

The mean differences in fertilization success may be attributed to variability among males. Lower fertilization success with fewer males was associated with higher variability in fertilization success among individuals (or groups of individuals). Variation among males in sperm characteristics (Casselman & Montgomerie, 2004) can affect fertilization success in addition to genetic incompatibilities between individual males and females (Zeh & Zeh, 1996, 1997; Wirtz, 1997). While all males in this study had motile sperm, they varied greatly in their ability to fertilize eggs. This might be partly due to the fact that while total milt volumes were kept equal during the experiment, potential differences in spermatocrit were not controlled for among males. The number of sperm in a given volume of milt can affect the probability of egg fertilization (Rosenthal *et al.*, 1988; Rakitin *et al.*, 1999), although they are not always directly correlated (Tvedt *et al.*, 2001) because other sperm characteristics such as sperm velocity can be stronger determinants of fertilization success (Gage *et al.*, 2004). Potential differences in sperm characteristics were not examined in this study. Additionally, it was decided *a priori* that controlling for equal amounts of milt from each male and equal amounts of sperm from each male could not both be done. Using spermatocrit to control for equal amounts of sperm would be a less direct test of the desired hypotheses. The design was such that any differences in spermatocrit among males will not bias the primary result. For example, if a single male had substantially lower spermatocrit than the others, it was consistent across all five treatments within that trial. Ideally, the best design would control for variability in the volume of milt that would be produced by naturally spawning males, and

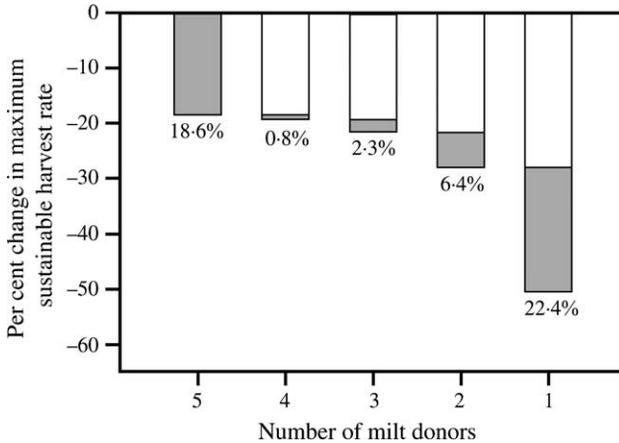


FIG. 5. Per cent change in the estimated maximum sustainable harvest rate from 100% fertilization, and with a change in fertilization success as the number of milt donors decreases. Grey shaded areas and corresponding numbers represent the per cent decline from the previous category.

address questions on the mechanisms involved by controlling for spermatocrit as well.

Omitting potential spermatocrit differences among males does not influence the conclusion about the effect of the number of males contributing milt, but is a possible cause of differences in fertilization success among individual males. When many males (four or five) were used there was a high probability that there was at least one male among them that could successfully fertilize a given female's eggs, resulting in all combinations having high fertilization success. When only one male was used, however, some individuals had high fertilization success and some very low, resulting in a lower mean fertilization success and high variability. The addition of another male's milt greatly reduced variability and increased the mean fertilization success among combinations. The observed difference in fertilization success with the quantity of males was partially due to the inclusion of a poor quality male in each trial (male 1 in trial 1, male 3 in trial 2 and male 5 in trial 3). The conclusion is unaffected as the purpose was to compare if male quantity affected fertilization success, not why it might differ. If these three males are removed from the analysis, the trend remains (mean fertilization success increases from 53% for the one male treatment to 64% for two males). Due to a large reduction in power (total sample size drops from 150 to 60), however, the trend is no longer statistically significant.

Assuming that this was a random sample of the males available to spawn (rainbow smelt are only in the stream for a few hours), and three of 15 did not achieve much fertilization success, it is possible that some females may encounter similarly unsuccessful males under natural spawning conditions. Even when different males successfully fertilize eggs, they can have a large effect on offspring characteristics. For example, Rideout *et al.* (2004) showed that hatching success, and aspects of larval size of haddock *Melanogrammus aeglefinus* (L.), were significantly influenced by paternity. In that study, some

morphological traits were influenced more by paternity than maternity. While many studies attempt to control for maternal effects, paternal effects on traits beyond fertilization success are not as heavily emphasized, although they should be included when measuring fitness components of offspring.

In designing the current experiment, enough milt had to be used from each male to ensure it could be accurately pipetted. Five per cent of the egg volume was chosen as the total amount of milt to use for each cross, which amounted to 2.5% of the fertilization medium. The conclusion assumes that the trend among treatments would remain the same, given any milt to egg dilution. In addition, the estimated reduction in maximum sustainable harvest rate from 100% fertilization success would differ if the overall success changed with the dilution.

The current study suggests that including males in population level processes may lead to more accurate estimates of intrinsic rates of increase. Taken further, these results can be important in the context of global fishery collapses (Hutchings, 2000; Myers & Worm, 2003). Reduced population size should decrease the number of spawning females, resulting in lower total egg production, and may lead to decreased total recruitment in the absence of density-dependent effects on egg and larval survival. Lower population size, however, will probably also reduce the abundance of males, and may decrease the number available to spawn with each female (the degree of which is unknown as a single male may fertilize eggs from several females over a spawning season). If fertilization success is related to the number of males involved in spawning, low population size may result in reduced egg production and egg fertilization success, which is an Allee effect (Levitan *et al.*, 1992). The results show that this can have an effect on the maximum sustainable harvest rate of a population. If the changes in fertilization success observed in the experiment occur in other species, it could exacerbate problems of low population sizes in collapsed fisheries. For example, one of the greatest declines in marine fishes has been the Atlantic cod stock off north-eastern Newfoundland (COSEWIC, 2003). Although fishing has been closed since 1992 the stock has not recovered (DFO, 2003), and Allee effects may be playing a role (Rowe & Hutchings, 2003). Experiments on captive Atlantic cod suggest that there may be an effect of the number of spawning males on overall fertilization success (Rowe *et al.*, 2004) similar to that seen here. In their experiment, Rowe *et al.* (2004) observed this reduction over many spawning events, suggesting that the number of males is indeed important, despite the fact that each male often spawns with more than one female. There is a dearth of information, however, on fertilization success in the wild.

In summary, the study suggests that the number of males participating in spawning events can influence fertilization success and have strong implications for population growth and recovery. While this is only one of many possible mechanisms involved, it indicates that considering fertilization success when calculating population level parameters may be more important than previously thought, particularly with respect to management of severely depleted populations.

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