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Sperm from pheromone primed brown trout (*Salmo trutta* L.) produce more larvae

Gustav Hellström[1] Prestegaard Tore[2], Dannewitz Johan[2] and K. Håkan Olsén[3]

[1]Department of Wildlife, Fish and Environmental Studies, Swedish University of Agricultural Sciences, Umeå, Sweden.

[2]Institute of Freshwater Research, Department of Aquatic Resources, Swedish University of Agricultural Sciences, Drottningholm, Sweden.

[3]School of Life Sciences, Södertörn University, SE 141 89 Huddinge, Sweden

Abstract

Male goldfish (*Carassius auratus*) exposed to female hormonal pheromones express increased milt volumes and their sperm fertilize more eggs than sperm from unprimed males. Ovulated salmonid females also release odours that increase volumes of strippable milt in males. It is, however, not known if the priming pheromones affect the ability of sperm to fertilize eggs in salmonids. In this study we compare the proportion of larvae produced from in vitro fertilization tests between primed brown trout *(Salmo trutta)* males exposed to a mix of female urine and ovarian fluids, and control males exposed only to 0.9 % sodium chloride. We also investigate priming effects on milt yield and sperm motility. Fertilization tests with sperm from single males, as well as sperm from two males (i.e. sperm competition), were performed. Primed males generated more larvae in both the single male and competition fertilization tests. No differences between treatments in milt yield and sperm motility could be established.

Introduction

Sex pheromones play an integral part in the reproduction of several species of fish (Liley 1982; Stacey and Sorensen 2006). Through the olfactory sense, fish detect and respond to scent released by conspecifics, resulting in elevated reproduction readiness in the receiving fish and in increased coordination of the spawning event (Stacey and Sorensen 2006). A lot of the information about sex pheromones in fish is based on studies of goldfish and various salmonids. In goldfish and the related crucian carp (*Carassius carassius*), the sex pheromones are closely connected to the production of sex hormones (Stacey and Sorensen 2002, 2006; Stacey et al. 2003). Rising blood concentrations of the steroid 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and prostaglandin $F_{2\alpha}$ (PGF2α) hormones indicates the reproduction readiness of the female, and the males has hence been found to detect these hormones and their metabolites at low concentrations (Sorensen et al. 1989; Bjerselius and Olsén 1993; Kobayashi et al. 2002). Exposure to 17,20β-P in the ambient water has priming effects on males of both goldfish and crucian carp, resulting in increased blood plasma levels of LH in mature males, followed by increased volumes of strippable milt (Bjerselius et al. 1995 a, b; Olsén et al. 2006) and ejaculate volumes (Hoysak and Stacey 2008). In goldfish, males exposed to 17,20β-P also have more active spermatozoa than unexposed males. Further, both in-vitro tests and spawning competition tests show that spermatozoa from primed males fertilized more eggs (DeFriapont and Sorensen 1993, Zheng et al. 1997).

In salmonids, female odours have been demonstrated to induce both behavioural effects and endocrine changes in males (Burnard et al. 2008). Water scented by ovulated females of rainbow trout (*Oncorhynchus mykiss*) attracted males, as well as triggered enhanced endocrine stimulation and milt availability (Emanule and Dodson 1979; Olsén and Liley 1993). Female urine and/or ovarian fluids are known to increase concentrations of sex hormones in Atlantic salmon and brown trout males (Olsén et al. 2000, 2001), a priming effect mediated through pheromones with prostaglandins such as PGF2α as an important part (Waring et al. 1996; Moore et al. 2002). In brown trout, PGF2α is present both in milt and ovarian fluid (Moore et al. 2002). In rainbow trout the identity of the active priming

molecule (or molecules) is not yet known, in spite of great efforts to isolate the compound (Scott et al. 1994).

Although studies confirm female odour priming effects in salmonid males, little is known whether primed males attain reproductive advantages in terms of higher fertilisation rates and production of larvae. In this study we investigate the effect of endocrine priming by female odours on the spermatozoa in mature brown trout parr. Hatching success is compared between primed and unprimed males in single and competition fertilization trials. Difference in milt availability and spermatozoa activity is also tested.

Materials and Methods

The study was conducted at the Fishery Research Station in Älvkarleby (N 60° 33.776', E 17° 26.597'), Swedish University of Agricultural Sciences, during the period 12th to 25th October 2006. The station farms Atlantic salmon and brown trout for stocking into the nearby river Dalälven, using returning adults caught in the river as breeders. The experiments were conducted on one year old mature brown trout male parr, hatched and reared at the station under standard farming conditions. A male was identified as mature if milt could be expressed from the gonopore after applying gentle pressure on the abdomen. Males were kept in two 400 l holding tanks continuously supplied with river water with a temperature between 10.5 and 12.0° C. Each tank was covered with a lid to reduce influence from surrounding activity.

Five hours before the *in vitro* fertilization tests, the males were transferred to two adjacent tanks identical to the holding tanks and exposed to 40 ml of female ovarian fluid and urine mix (OVF) (Olsén et al. 2000) or the same amount of 0.9 % NaCl (control fish). OVF was collected the same morning from several ovulating female trout and kept on ice until added to the tanks. 250 l of standing water was used in both the exposure tanks and the control tanks. The water was aerated during the exposure to OVF.

In vitro fertilization experiments

After the 5 hours exposure period, the fish were transported to a nearby laboratory for fertilization tests. Anaesthetized fish (0.05% 2-phenoxyethanol) were placed upside down in a moist slotted foam pad. Milt was collected by gently squeezing the abdomen from the midventral region posterior to the gonopore between moistened thumb and finger, and collected in a glass tube. When all the available milt was emptied, the male was killed by an overdose of phenoxyethanol. The milt was stored in tubes and kept on ice. Care was taken to avoid influence of urine in the milt-samples. Eggs from ovulated females were collected approximately 7 hours before the fertilization tests and kept in 8°C 0.9% NaCl until fertilization.

Two different in vitro fertilization tests were conducted. Eggs from a single female were fertilized by 1) sperm from a single male and 2) sperm from two males, one primed and one unprimed. The fertilization protocol began with the placement of twenty eggs in the center of a dry glass beaker (1000 ml). A small amount of milt was then pipetted into one spot close to the wall of the beaker. If sperm from two males were used, their milt was placed at separated spots at equal distance from the eggs. 20 ul of packed sperm per male was used in each fertilization trial. To ensure this amount of sperm to be equal for all males, the volume of milt added to the beaker was calculated based on individual male spermatocrit, following the protocol of Winnicki and Tomasik (1976). Milt was drawn up in microhematocrit tubes which were sealed and centrifuged for 5500 x g for 5 minutes. The amount of milt used for an individual male was calculated from the average of two spermatocrit samples.

After the addition of milt, 400 ml of aerated groundwater was flushed into the beaker, ensuring complete mixing of sperm and eggs. The beaker was then left alone for three minutes, whereafter the eggs was carefully poured into a small colander and gently washed before being placed in a specially designed incubator (Olsén and Winberg 1996). Each egg clutch was kept in a separate container in the incubator, each container having its own supply of aerated ground water (7.8 ± 0.9° C), hence reducing any contact with

egg clutches in neighboring containers (Olsén and Winberg 1996). Incubating eggs were kept dark. Dead eggs were regularly removed throughout the incubation period, up until hatching. Number of hatched and dead eggs was counted for each clutch. According to Elliott (1994) brown trout eggs hatch after 444 day degrees (°d). This study was terminated after 480 °d whereupon all remaining unhatched eggs were inspected and concluded dead/unfertilized. All hatched fry from the two-male fertilization tests were killed by an overdose of phenoxyethanol and stored in 70% ethanol for subsequent parentage testing.

Parentage analyses

Genotyping of parents and fry was conducted at the Swedish National Board of Fisheries Research Station in Drottningholm, Stockholm, using 12 microsatellites. For detailed information on the microsatellites used, the protocols for DNA-extraction and PCR-amplification, and allele size determination, see Dannewitz et al. (2003) and Dannewitz et al. (2004). Based on allele comparison between potential parents and offspring, using the software WhichParents (written by Will Eichert, Bodega Marine Laboratory, Bodega Bay, CA; available on http://bml.ucdavis.edu/research/research-programs/conservation/salmon-research/salmon-genetics-software/), it was possible to unambiguously assign 182 (94%) of all fry. Twelve individuals (6%) matched more than one possible father and were hence omitted from further analysis.

Sperm motility

Immediately after the fertilization tests, the samples of milt were transferred to an adjacent lab for sperm motility tests. All samples were on ice throughout the tests. The sperm were activated by pipetting 20 ul of milt from the sample-tube into an eppendorf-tube filled with 8 ml of water. Instantly after activation, 4 ul of the milt/water mix was pipetted onto a coverslip placed under a microscope (x100 magnification). A specially designed, electricity driven, cooler-system was integrated into the microscope to keep the temperature of the coverslip to 5°C. A video camera was attached to the ocular train of

the microscope, filming the focal plane of exposure. The time from activation until the last spermatozoa stopped moving, was recorded on a VCR. The number of motile and non-motile spermatozoa was counted at 30, 40 and 50 seconds after activation. Movements of fluids prevented an accurate count earlier than 30 seconds after activation. Counting was done on a TV-screen divided into sections, using the video-recordings from the video camera. Two motility tests were conducted per male milt sample.

Body total length (mm) and body weight (g) was recorded for all fish used in the study. The weight of the testicles and the total amount of available, strippable milt were measured for each male. Gonadosomatic index (GSI) was calculated as the percentage of the weight of the testes to the body weight. Fin-clips from all males and females were collected and stored in 70% ethanol for subsequent genotyping.

Analyses

Fertilization tests

For the single male fertilization tests, the number of hatched and dead/unfertilized eggs per clutch was analyzed as proportions in a logistic regression using a generalized linear mixed effects model (GLMM) with binomial errors (Bates and Maechler 2010). Priming by OVF was treated as two-level fixed effect (yes/no). Female was added as a random effect to account for potential pseudoreplication. The model was overdispersed and this was corrected for by adding a random effect for every level of observation. Significance of priming was determined by a log-likelihood test comparing models with and without the term, assuming chi-squared distribution of the ratio-statistic.
Variance components were calculated to assess the influence of between female variation on the overall variation.

For the two male sperm competition tests, the ratio of hatched fry belonging to either of the two fathers (primed vs unprimed) was analyzed using Pearson's Chi-squared test.

Sperm-motility

The average proportion of motile spermatozoa over the two runs was analyzed as a function of Priming by OVF and Time using a generalized linear model (GLM). Priming was treated as a two-level fixed effect (yes/no) and time as an ordinal three level fixed effect (30, 40 and 50). An interaction term between priming and time was included. Quasi-binomial errors were used to correct for overdispersion and F tests were used for significance testing (Crawley 2007).

Size, GSI and milt yield of males

Differences in weight, length, GSI, milt yield and spermatocrit between groups of primed and unprimed males in both the single male and the sperm competition fertilization tests were assessed using one-way analysis of variance. Milt-yield was log transformed to acquire normality.

All analyzes were preformed in the statistical program R (R Development Core Team 2010).

Results

Overall, 50 separate fertilizations trials were conducted, of which 40 were single male fertilization and 10 were sperm competition tests. In total, 40 males were used, 20 primed and 20 unprimed. Eggs from seven different females were used, although each fertilization trial was performed only with eggs from a single female.

Fertilization tests

In the single male fertilization tests, primed males had significantly higher probability of hatching success (Mean ± SE; 0.304 ± 0.051) than unprimed males (0.185 ± 0.037)

($\chi^2(1)$=5.32; p = 0.021, figure 1). Based on variance components, differences between females explained 72.2 % of the overall variance. The unexplained (residual) variance was 27.8%.

In the sperm competition tests, significantly more offspring belonged to primed males (70.1%) compared to unprimed males (29.9%) ($\chi^2(8)$ = 18.1, p = 0.020, figure 2).

Sperm motility

Time had a significant effect on the probability of motile sperm ($F_{2,162}$=10.8, p<0.01, figure 3). There were significantly more motile spermatozoa after 30 seconds than after 50 seconds (contrast analysis, p<0.01). There were also significantly more motile spermatozoa after 40 seconds compared to 50 seconds (contrast analysis, p<0.01). Neither Priming nor the interaction between Priming and Time had any effect on the proportion of motile spermatozoa (p>0.05).

Differences in size, GSI, milt yield and spermatokrit

There were no significant differences in length, weight, GSI, amount of milt or spermatokrit between primed and unprimed males in the single male fertilization tests and between primed and unprimed males in the two male sperm competition tests (one-way analysis of variance, p>0.05, table 1)

Discussion

The present study demonstrates that spermatozoa from male brown trout that have been exposed to odours from ovulated females generated a higher proportion of hatched eggs and larvae than sperm from males without exposure. Similar results have been found in goldfish, where males primed with the hormonal pheromone 17,20β-P had higher

fertilization rates, both *in vitro* and *in vivo*, compared to males that had not been exposed to the pheromone (De Fraipont and Sorensen 1993; Zheng et al. 1997; Hoysak and Stacey 2008).

The underlying mechanism behind this effect remains unclear. Priming pheromones are known to increase milt production and hence the total number of spermatozoa, in both goldfish and salmonid males (Stacey 2011; Olsen and Liley 1993), although the concentration of sperm (estimated using spermatocrit) did not increase in the salmonids (Olsén and Liley 1993). Under natural spawning conditions for an external fertilizer such as salmonids, higher number of spermatozoa may be an advantage in the competition for fertilization (Petersen & Warner 1998). In this study, we could not detect any difference in milt yield, nor spermatocrit, between primed and unprimed males. Potentially, this may be a consequence of to short exposure time, making sufficient difference in milt production not able to arise, or due to low concentration of female odour during exposure. However, Moore et al (2002) found elevated milt volumes in brown trout exposed to female urine using the same duration of exposure as this study (5 hours), but lower concentration of female odour (10^{-5} vs. $\sim 10^{-4}$). That study was conducted under similar laboratory conditions as this study, although in slightly lower temperatures (6-8° C). In this study, any potential difference in sperm density was accounted for by adjusting milt volumes for each male to the spermatocrit, making the number of sperm used in the fertilization trials equal for both unprimed and primed males.

Several studies report aspects of spermatozoa motility (e.g. proportions of motile sperm, swimming speed, etc) to influence fertilization success (Liljedal et al. 2008; Rurangwa 2004). Direct exposure to ovarian fluids, occurring naturally during the spawning event, is known to enhance spermatozoa motility and longevity (Turner and Montgomerie 2002; Diogo 2010). In this study, however, difference in the exposure to ovarian fluids occurred before the spermatozoa were activated. During the fertilization trials, primed and unprimed spermatozoa were treated identically, hence being exposed to the same amount of any potential ovarian fluids coming with the eggs. Direct effects of ovairan fluids on

activated spermatozoa could hence not explain differences in fertilization rates shown in our study.

The proportion of active (motile) sperm did not differ between primed and unprimed males in this study. A few studies have concluded priming effects on sperm motility. Studies on goldfish have shown that males primed with the hormonal pheromone 17,20β-P have more active sperm compared to males that had not been exposed to the pheromone (De Fraipont and Sorensen 1993; Zheng et al. 1997; Hoysak and Stacey 2008; Stacey 2011). Similar tendencies were found by Liley et al. (2002) for rainbow trout, where adult males exposed to nesting females had a greater proportion of active sperm compared to unexposed males kept in all-male groups, although the difference was not significant. Differences in fertilization rates could however not be detected in that study (Liley et al. 2002).

The testing procedure did not allow us to reliably quantify sperm motility before 30s after activation, due to movement of the water placed on the coverslip. The swimming activity during the initial 5 -10 s after activation can be crucial to the outcome of sperm competition. In tests with rainbow trout, over 80% of 50-100 eggs were fertilized within the first 10s, using the same sperm concentration as in our study (Liley et al. 2002). It is possible that the >30s motility measured in this study does not accurately reflect potentially important differences in motility occurring during the first 10s. Furthermore, we did not measure the swimming speed and trajectory pattern of the sperm, characteristics that very well may influence fertilization success.

The osmotic pressure, chemical composition and pH of seminal plasma have great influence on the activity and longevity of sperm (review Knapp and Carlisle 2011). Exposure to female stimuli, such as priming pheromones or nest digging, increase the production and release of the progestin 17,20β-P from the testes in salmonid males (e.g. Olsén and Liley 1993). 17,20β-P is important for the final maturation of sperm, the release of sperm to the sperm ducts, the increase in milt volume and fluidity, the pH of the seminal fluid and sperm motility (Miura et al. 1992; Pankhurst 2008; Scott et al.

2010; Knapp and Carlisle 2011). It has been suggested that membrane bound receptors and/or genomic progestin receptors are important to increase the activity of sperm in fish (Thomas 2003; Pankhurst 2008). Tubbs and Thomas (2009) exposed sperm from Atlantic croaker (*Micropogonias undulates*) to the maturation progestin 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S). 20β-S is bound to membrane receptors (mPRα) on the sperm mid piece and flagella. The exposure stimulated sperm hyper-motility in Atlantic croaker (*Micropogonias undulates*) (Tubbs and Thomas 2009), increasing the percentage of hypermotile sperm as well as improved fertilization rates. The hypermotility was also associated with activation of an olfactory G protein, co-expressed with mPRα. Fish treated with LHRH increased mPRα expression in sperm and induced hyper motility. Thus, increased release of 17,20β-P after female odour priming may increase sperm swimming activity through direct effect on sperm. This possibility should be addressed in future studies.

Probability of hatching was comparatively low (<35%) in this study compared to what is commonly achieved in commercial hatcheries. This is likely due to the low concentration of sperm used in the fertilization trials. We deliberately chose low concentrations to amplify the competition aspect, and to distinguish characteristics of the single sperm rather than the amount of sperm.

Although variance component analysis revealed considerable variation between females in the probability of hatching success, female variation did not influence effective comparisons between treatments as the use of eggs from different females were equally balanced over all tests. Quality of eggs and ovarian fluids may vary greatly between individual females (Brooks et al. 1997; Urbach et al. 2005), and there seems to be differences in the effect of ovarian fluids on spermatozoa activity depending on which individuals are combined (Urbach et al. 2005; Wojtczak et al. 2007). This underlies the importance of using several females in fertilization tests.

In the present study we demonstrate that sperm from precocious brown trout males exposed to odours from ovulated females generated a higher probability of hatched eggs and larvae compared to sperm from males receiving no priming. Future studies should address underlying biochemical mechanisms behind such priming effects.

References:

Bates, D., Maechler, M., 2010. lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-37. http://CRAN.R-project.org/package=lme4

Bjerselius, R., Olsén, K.H., 1993. A study of the olfactory sensitivity of crucian carp (Carassius carassius) to 17α,20β-dihydroxyprogesterone and prostaglandin F2α. Chem. Senses 18, 427-436.

Bjerselius, R., Olsén, K.H., Zheng, W., 1995a. Endocrine, gonadal and behavioural responses of male crucian carp (*Carassius carassius*) to the hormonal pheromone 17α, 20β-dihydroxy-4-pregnen-3-one. Chem. Senses 20, 221-230.

Bjerselius, R., Olsén, K.H., Zheng, W., 1995b. Behavioural and endocrinological responses of mature male goldfish to the sex pheromone 17α, 20β-dihydroxy-4-pregnen-3-one in the water. J. Exp. Biol. 198, 747-754.

Brooks S, Tyler CR, Sumpter JP., 1997. Egg quality in fish: what makes a good egg? Reviews in Fish Biology and Fisheries. 7:4, 387-416.

Burnard, D., Gozlans RE., Griffiths SW., 2008. The role of pheromones in freshwater fishes. Journal of Fish Biology 73, 1–16

Crawley, M., 2007. The R Book, John Wiley & Sons, Chichester.

Dannewitz, J., Petersson, E., Dahl, J., Prestegaard, T., Löf, A.C., Järvi, T., 2004. Reproductive success of hatchery-produced and wild-born brown trout in an experimental stream. J. App. Ecol. 41, 355–364.

Dannewitz, J., Petersson, E., Prestegaard, T., Järvi, T., 2003. Effects of sea-ranching and family background on fitness traits in brown trout *Salmo trutta* reared under near-natural conditions. J. App. Ecol. 40, 241–250.

DeFraipont, M., Sorensen, P.W., 1993. Exposure to the pheromone 17α, 20β-dihydroxy-4-pregnen-3-one enhances the behavioural spawning success, sperm production, and sperm motility of male goldfish. Anim. Behav. 46, 245-256.

Diogo P., Soares1, F., Dinis M.T., Cabrita E., 2010. The influence of ovarian fluid on Solea senegalensis sperm motility. J. Appl. Ichthyol. 26, 690–695

Elliott, J.M. 1994., Quantitative ecology and the brown trout, Oxford University Press, Oxford, New York.

Emanuel, M.E., Dodson, J.J., 1979. Modification of th erheotropic behaviour of male rainbow trout (*Salmo gairdneri*) by ovarian fluid. J. Fish. Res. Board. Can. 36, 63-68.

Hoysak, D.J., Stacey, N.E., 2008. Large and persistent effect of a female steroid pheromone on ejaculate size in goldfish *Carassius auratus*. J. Fish Biol. 73, 1573-1584.

Knapp, R. Carlisle, S.L., 2011. Testicular function and hormonal regulation in fishes. In: Norris, D.O., Lopez, K.H. (Eds). Hormones and Reproduction of Vertebrates. Vol. 1 - Fishes. Academic Press, New York, pp. 43-63.

Kobayashi, M., Sorensen, P.W., Stacey, N.E., 2002. Hormonal and pheromonal control of spawning behaviour in the goldfish. Fish. Physiol. Biochem. 26. 71-84.

Liley, N.R., 1982. Chemical communication in fish. Can. J. Fish. Aquat. Sci. 26, 3-6.

Liley, N.R., Tamkee, R., Tsai, R., Hoysak, D.J., 2002. Fertilization dynamics in rainbow trout (*Oncorhynchus mykiss*): effect of male age, social experience, and sperm concentration and motility on in vitro fertilization. Can. J. Fish. Aquat. Sci. 59, 144-152.

Liljedal, S., Rudolfsen, G., Folstad, I., 2008. Factors predicting male fertilization success in an external fertilizer. Behav Ecol Sociobiol 62:1805–1811

Miura, T., Yamauchi, K., Takhashi, H. and Nagahama, Y., 1991. The involvement of steroid hormones in gonadotropin-induced testicular maturation in male Japanese eel (Anguilla japonica). Biomed. Res. 12, 241–248.

Moore, A., Olsén, K.H., Lower, N., Kindahl, H., 2002. The role of F-series prostaglandins as reproductive priming pheromones in the brown trout. J. Fish. Biol. 60, 613-624.

Olsén, K.H., Bjerselius, R., Mayer, I., Kindahl, H., 2001. Both ovarian fluid and female urine increase sex steroid levels in mature Atlantic salmon (*Salmo salar* L.) male parr. J. Chem. Ecol. 27, 2337-2349.

Olsén, K.H., Bjerselius, R., Petersson, E., Järvi, T., Mayer, I., Hedenskog, M., 2000. Lack of specific primer effects of odours from female Atlantic salmon (*Salmo salar* L.) and brown trout (*Salmo trutta* L.). Oikos 88, 213–220.

Olsén, K.H., Liley, N.R., 1993. The significance of olfaction and social cues in milt availability, sexual hormone status and spawning behaviour of male rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 89, 107-118.

Olsén, K.H., Sawisky, G.R., Stacey, N.E., 2006. Endocrine and milt responses of male crucian carp (*Carassius carassius* L.) to periovulatory females under field conditions. Gen. Comp. Endocrinol. 149, 294-302.

Olsén, K.H., Winberg, S., 1996. Learning and sibling odour preference in juvenile Arctic char, *Salvelinus alpinus* (L.). J. Chem. Ecol. 22, 773-786.

Pankhurst, N.W., 2008. Gonadal steroids: functions and patterns of change. In: Fish Reproduction. Rocha, M.J., Arukwe, A., Kapoor, B.G. (Eds.). Science Publishers, Enfield NH, pp. 67-111.

Petersen C.W & Warner RR., 1998. Sperm competition in Fishes. In: Birkhead TR & Moller AP. (Eds). Sperm competition and sexual selection. Academic, San Diego.

R Development Core Team, 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Rurangwaa, E., Kimeb, D.E., Olleviera, F., Nasha J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234, 1-28

Scott, A.P., Sumpter, J.P. Stacey, N.E., 2010. The role of the maturation-inducing steroid 17, 20β-dihydroxypregn-4-en-3-one, in male fishes: a review. J. Fish Biol. 76, 183-224.

Scott, A.P., Liley, N.R., Vermeirssen, E.L.M., 1994. Urine of reproductively mature female rainbow trout, *Oncorhynchus mykiss* (Walbaum), contains a priming pheromone which enhances plasma levels of sex steroids and gonadotrophin II in males. J. Fish Biol. 44, 131-147.

Sorensen, P.W., Chamberlain, K.J., Stacey, N.E., 1989. Differing behavioural and endocrinological effects of two female sex pheromones on male goldfish. Horm. Behav. 23, 317-332.

Stacey, N.E., 2011. Hormonally derived sex pheromones in fishes. In: Norris, D.O., Lopez, K.H. (Eds). Hormones and Reproduction of Vertebrates. Volume 1 - Fishes. Academic Press, New York, pp. 169-192.

Stacey, N.E., Chojnacki, A., Narayanan, A., Cole, T.B., Murphy, C.A., 2003. Hormonally-derived sex pheromones in fish: exogenous cues and signals from gonad to brain. Can. J. Physiol. Pharmacol. 81, 329-341.

Stacey, N.E., Sorensen, P.W., 2006. Reproductive pheromones. In: Sloman, K.A., Wilson, R.W., Balshine, S. (Eds.), Fish Physiology: Behaviour and Physiology of Fish, Vol. 24, Elsevier Inc, San Diego, pp. 359-412.

Stacey, N.E., Sorensen, P.W., 2002. Fish hormonal pheromones. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin T. (Eds), Hormones, Brain and Behaviour, Vol. 2, Academic Press, New York, pp. 375-435.

Thomas, P., 2003. Rapid, nongenomic steroid actions initiated at the cell surface: lessons from studies with fish. Fish Physiol. Biochem. 28, 3-12.

Turner, E., Montgomerie, R., 2002. Ovarian fluid enhances sperm movement in Arctic charr. Journal of Fish Biology 60, 1570–1579

Tubbs, C., Thomas, P., 2009. Progestin Signaling through an Olfactory G Protein and Membrane Progestin Receptor-α in Atlantic Croaker Sperm: Potential Role in Induction of Sperm Hypermotility. Endocrinology 1, 473-484

Urbach, D., Folstad, I., Rudolfsen, G., 2005. Effects of ovarian fluid on sperm velocity in
Arctic charr (*Salvelinus alpinus*). Behav Ecol Sociobiol 57:438-444

Waring, C.P., Moore, A., Scott, A.P., 1996. Milt and endocrine responses of mature male
Atlantic salmon (*Salmo salar* L.) parr to water-borne 17α, 20β-dihydroxy-4-
pregnen-3-one-20-sulfate, and the urines from adult female and male salmon. Gen.
Comp. Endocrinol. 103, 142-149.

Winnicki, A., Tomasik, L., 1976. Spermatocrit as a method for biological evaluation of
fish sperm, Acta Ichthyol. Piscator. 6, 3–8.

Wojtczak, M., Grzegorz, J., Dietrich, A., Słowińska, M., Dobosz, S., Kuźmiński, H.,
Ciereszko, A., 2007. Ovarian fluid pH enhances motility parameters of rainbow
trout (*Oncorhynchus mykiss*) spermatozoa. Aquaculture 270, 259–264.

Zheng, W., Strobeck, C., Stacey, N.E., 1997. The steroid pheromone17α, 20β-dihydroxy-
4-pregnen-3-one increases fertility and paternity in goldfish. J. Exp. Biol. 200,
2833-2840.

TABLES:

Table 1. Mean ± standard deviation of weight, length, gonadosomatic index, milt yield
and spermatokrit for Primed and Unprimed males in the single male fertilization tests and
the two male sperm competition tests. Primed and unprimed males did not differ
significantly in any of the variables (one-way analysis of variance tests, $p>0.05$).

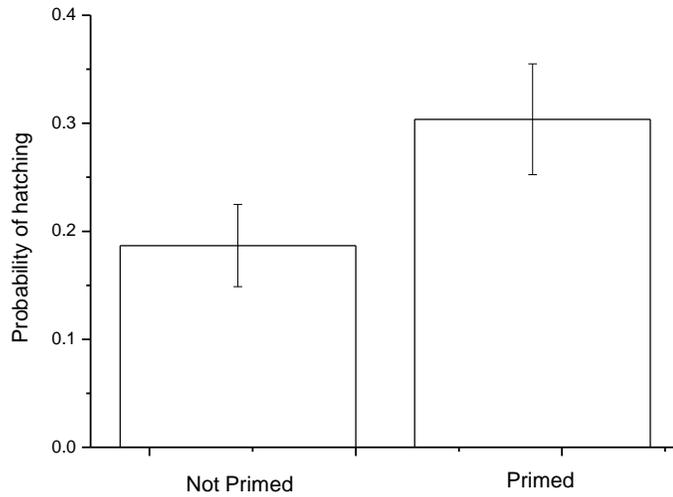|	Weight (g)	Length (mm)	GSI	Milt (g)	[Spermatokrit]
Single male fertilization					
Primed (n=20)	93.3 ± 22.7	208.2 ± 10.2	2.52 ± 0.78	1.51 ± 0.54	0.62 ± 0.11
Unprimed (n=20)	99.4 ± 16.5	203.3 ± 16.4	2.52 ± 0.840	1.43 ± 0.62	0.58 ± 0.13
Sperm Competition					
Primed (n=10)	95.5 ± 13.2	206.0 ± 8.5	2.56 ± 0.70	1.52 ± 0.71	0.62 ± 0.28
Unprimed (n=10)	91.7 ± 23.3	202.3 ± 15.6	2.67 ± 0.82	1.54 ± 0.52	0.59 ± 0.75

FIGURES

Figure 1. Probability (Mean ± CI) of hatching success for eggs fertilized by primed (n=20) and unprimed (n=20) males. Primed males had a significantly higher probability of hatching success compared to unprimed males.
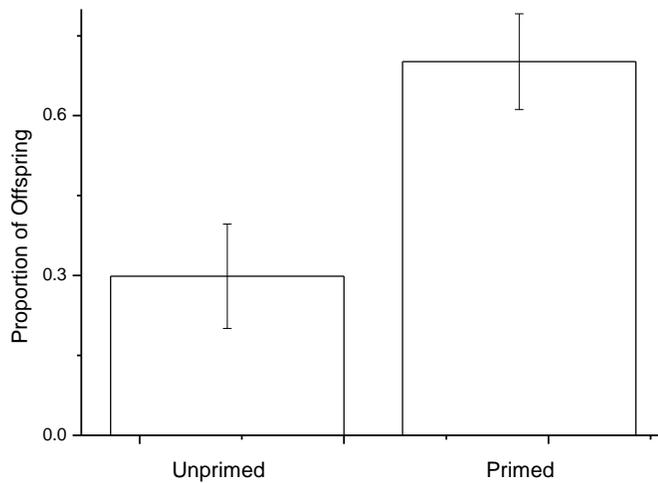

Figure 2: Mean proportions of assigned offspring from sperm competition fertilization trials (n=10) between primed and unprimed males. Significantly more larvae were assigned to primed males than unprimed males.
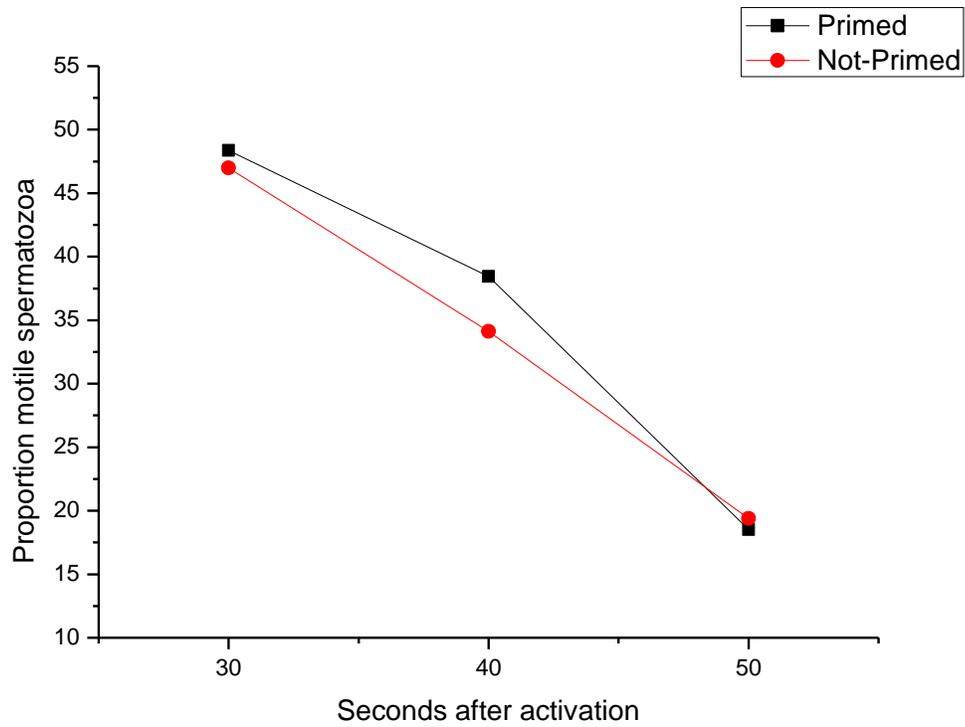
Figure 3. Probability of motile spermatozoa 30, 40 and 50 seconds after activation for primed and unprimed precocious males. There were no significant differences in overall probability between primed and unprimed males. Time had a significant effect on proportion of motile spermatozoa.