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Effects of progesterone on sperm motility in fathead minnow (*Pimephales promelas*)

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ABSTRACT

The steroid hormone progesterone (P4) is found at relatively high concentrations (~300 ng/L) in association with concentrated animal feeding operations (CAFOs). In an effort to better understand the potential endocrine disrupting effects of P4 in male fish, computer assisted sperm analysis (CASA) was used to evaluate the effects of this steroid on sperm motility in the fathead minnow (*Pimephales promelas*). The rationale for focusing on sperm motility is that certain progestins have been shown to bind to surface membrane receptors on fish spermatozoa and increase sperm swimming velocity. It was hypothesized, therefore, that sperm swimming velocity might be a useful indicator of progestin exposure in fish. Adult male fathead minnows (ages 6–12 months) were exposed to environmentally relevant doses of P4, both longer-term (1 week, *in vivo* exposure) and short-term (minutes, *in vitro* exposure). Sperm were then video recorded and analyzed by CASA. When fathead minnows were continuously exposed for 1 week to low levels of progesterone *in vivo* there was a significant dose-dependent reduction in sperm motility. There was no effect of short-term P4 exposure on fathead minnow sperm swimming characteristics. Additional research is required to elucidate the mechanism by which progesterone alters sperm swimming in the fathead minnow. With further validation, the fathead minnow sperm motility assay may be a useful tool to rapidly screen for endocrine disrupting chemicals in the aquatic environment.

1. Introduction

More than 500 million tons of animal manure is produced annually in the U.S. from concentrated animal feeding operations (CAFOs). Both natural and synthetic steroids and their metabolites have been detected in runoff and soil samples from CAFOs (Durhan et al., 2005; Lange et al., 2002; Schiffer et al., 2001; Soto et al., 2004). In a recent survey of the steroid hormones associated with CAFOs in southern Wisconsin, researchers from the Wisconsin State Laboratory of Hygiene detected progesterone (P4) at concentrations as high as 350 ng/L in spring runoff (unpublished). This finding is compatible with reports that progestins can be excreted at high concentrations from hormone-implanted cattle, and that this class of steroid hormone can persist in the soil for several months (Schiffer et al., 2001).

Compared to androgens and estrogens, very little is known about the role of P4 as an endocrine disrupting chemical (EDC) in fish (Scott et al., 2010). Progestins mediate the process of final gamete maturation (spermiation) in male fish (Scott et al., 2010), and in some fish species progestins can bind to surface membrane receptors on mature spermatozoa and directly stimulate an increase in sperm motility (Thomas et al., 2004; Tubbs and Thomas, 2008, 2009). Thus, it was hypothesized that exposing fish to P4 from CAFO effluent could have a significant impact on the development, maturation and function of fish spermatozoa. Computer assisted sperm analysis (CASA) is an effective method to quantify changes in fish sperm swimming characteristics, and has been used in a wide variety of fish species for this purpose, including zebrafish, lake sturgeon, carp, bluegill, rainbow trout, and arctic char (Burness et al., 2004; Lahnsteiner et al., 2005; Linhart et al., 1995; Rudolfsen et al., 2006; Runnalls et al., 2007; Toth et al., 1995; Wilson-Leedy and Ingermann, 2007). To our knowledge, there have been no investigations on the impact of P4 on sperm swimming motility in fish.

The present study was conducted to evaluate the effects of P4 on spermatozoa swimming characteristics in male fathead minnows – a fish species widely used in ecotoxicological investigations. The primary objectives were to (1) optimize a CASA protocol for use with fathead minnows based on a protocol originally developed

Abbreviations: P4, progesterone; CASA, computer assisted sperm analysis; VSL, straight line velocity; VCL, curvilinear velocity; VAP, average path velocity; PMOT, percent motility; PLIN, percent linearity; CAFO, concentrated animal feeding operation.

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for zebrafish (Wilson-Leedy and Ingermann, 2006), and (2) use this optimized fathead minnow CASA procedure to evaluate the dose-dependent effects of long-term *in vivo* (7 days) exposure to environmentally relevant concentrations of P4 on fathead minnow sperm motility.

2. Materials and methods

2.1. Chemicals and stock solutions

The progesterone was purchased from Steraloids, Inc. (Newport, RI). All other chemicals were of the highest purity and purchased from Sigma Chemical Company (St. Louis, MO). Concentrated steroid stock solutions were prepared in 100% ethanol and diluted as required in distilled water. Ethanol concentrations in the final test solutions were less than or equal to 0.04%. Semen extender was prepared according to the method of Runnalls et al. (2007).

2.2. Fish

Reproductively mature male fathead minnows (6–12 months of age) were housed in 25-gallon tanks with flow-through water at 24–26 °C. The photoperiod was 16L:8D. Light intensity was ambient laboratory level (540–1080 lux). The fish were fed live and frozen artemia supplemented with formulated diet (flake feed and 45% protein salmon starter diet). Feeding frequencies and levels were adjusted as the fish grew (Denny, 1987; Parrott et al., 2006).

2.3. Semen preparation

Males were sacrificed and their testes surgically removed. Each testis was placed into a 1.5 ml centrifuge tube containing 1 ml of semen extender. The tube was incubated in a water bath at 24 ± 2 °C for 45 min after which time the testis was diced within the tube using a scalpel. The minced tissue preparation was vortexed for 2 s to release the mature spermatozoa. The tube was inverted several times to mix the semen and extender. Extended sperm preparations were used within 5 h of sacrificing the male.

2.4. Sperm activation and video recording

Ten microliters of the sperm mixture was pipetted into a 12 mm × 75 mm test-tube. Forty microliters of water was then added to a test tube and vortexed for 2 s to activate the sperm. Some tubes received a 0.8 mM NaCl solution that prevents fathead minnow sperm from becoming motile (Wilson-Leedy et al., 2009). This saline treatment served as a positive control.

Two microliters of the activated sperm mixture was pipetted onto a multiwell microscope slide, and covered with a glass cover slip. The sperm were observed using a Nikon (Melville, NY) Microphot-FX microscope (200× magnification) equipped with a Hamamatsu (Bridgewater, NJ) CCD video camera.

There was initially considerable drift as the sperm mixture was drawn by capillary action under the cover slip. This interfered with the CASA analysis of sperm motility. Various methods were tried to reduce drifting including using lesser volume and slower pipetting rates, but these did not alleviate the problem. Therefore, the video recordings were initiated 80 s after sperm activation, a time when drift had completely subsided. Video images were recorded for 15 s (i.e., video was recorded between 80 and 95 s after sperm activation). The video recordings were saved onto a hard drive and later analyzed using CASA according to Kime et al. (2001).

Table 1
Input parameters for Image J adapted for fathead minnows.

Input parameters	Input values
A. Minimum sperm size (pixels)	5
B. Maximum sperm size (pixels)	50
C. Minimum track length (frames)	16
D. Maximum sperm velocity between frames (pixels)	20
E. Minimum VSL for motile (μm/s)	3
F. Minimum VAP for motile (μm/s)	5
G. Minimum VCL for motile (μm/s)	15
H. Low VAP speed (μm/s)	5
I. Maximum percentage of path with zero VAP	1.0
J. Maximum percentage of path with low VAP	25
K. Low VAP speed 2 (μm/s)	10
L. Low VCL speed (μm/s)	15
M. High WOB (percent VAP/VCL)	80
N. High LIN (percent VSL/VAP)	80
O. High WOB two (percent VAP/VCL)	200
P. High LIN two (percent VSL/VAP)	200
Q. Frame rate (frames per second)	16
R. Microns per 1000 pixels	2930
S. Print *xy* co-ordinates for all tracked sperm?	0
T. Print motion characteristics for all motile sperm?	1
U. Print median values for motion characteristics	0

2.5. Analysis of sperm swimming parameters

The sperm were analyzed using the software program "Computer Assisted Sperm Analysis" (CASA), which is a freeware plug-in of the program Image J (Wilson-Leedy and Ingermann, 2006). The program can be downloaded at http://rsb.info.nih.gov/ij/. The CASA program tracks sperm individually and quantifies specific swimming characteristics including: percent motility (PMOT), curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), and percent linearity (PLIN). These parameters are defined in Wilson-Leedy and Ingermann (2007). In previous investigations, all of these parameters have been shown to correlate with fish fertilization success (Rurangwa et al., 2001).

The CASA input parameters used in the present investigation are shown in Table 1, and were based on those published for zebrafish spermatozoa by Wilson-Leedy and Ingermann (2006). These parameters can be used in all future studies using CASA to evaluate fathead minnow sperm motility, although specific parameters (*A*, *B*, *D*, *Q* and *R*) will require adjustment depending on what microscope and video recording equipment is used by different investigators. For example, in the current study, the video recording frame rate (*Q*) was 16 frames per second, and this value would need to be changed for video recordings made at a different frame rate. The input parameters *A*, *B*, *D* and *R* also need to be adjusted for different microscope magnification levels as these parameters are based on pixel number, not actual sperm size. All of the other CASA input values listed in Table 1 specify characteristics of fathead minnow sperm and do not need to be changed.

CASA uses two selection criteria to identify motile sperm. First, the program identifies slow moving sperm by ensuring that they meet minimum VSL, VCL and VAP requirements. Second, all sperm that pass the first screen are further analyzed to determine if they are moving autonomously or due to bulk water flow or drift. This is accomplished by ensuring that the sperm meet at least one of the following conditions: (1) they are not moving in a perfectly straight line (values less than the set points for parameters M, N, J and I), (2) they are moving faster than drifting sperm (values more than set points for parameters L and K), or (3) they are moving with a high degree of path curvature (values less than set points for parameters O and P). See Wilson-Leedy and Ingermann (2006) for a complete description.

Sperm motility and sperm curvilinear velocity both increased with the addition of 0.8 mM NaCl solution. These results were sim-