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EFFECTS OF THE PHYTOSTEROL STIGMASTANOL ON EARLY LIFE-STAGES AND LIFE-CYCLES OF FATHEAD MINNOWS (*Pimephales promelas*) AND *Ceriodaphnia dubia*

TECHNICAL BULLETIN NO. 788

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Acknowledgments

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PRESIDENT'S NOTE

Questions recently posed by investigators from several countries have focused on the possible effects of pulp mill effluents on fish reproduction. NCASI's Aquatic Biology Program has addressed these questions using fathead minnow life-cycle tests, experimental streams, and more recently, long-term receiving-water studies. One of the goals of these studies is to determine effluent components and their sources that have the potential to alter fish reproduction at concentrations found in biologically treated final effluents. One approach to achieving this goal has been to measure compounds or components in effluents during laboratory fathead minnow life-cycle tests, and to compare the concentration of each component with fish reproduction endpoints.

In previous studies, fish reproduction endpoints from six of NCASI's laboratory fathead minnow life-cycle tests with effluents were compared to concentrations of compounds found in the effluents. Five of the six tests used effluents from kraft mills with: 1) OD + ECF bleaching, 2) ECF bleaching, 3) OD + 70% substitution bleaching, 4) 90% Cl_2 + 10% ClO_2 bleaching, and 5) no bleaching. The sixth test used effluent from an OCC recovered fiber mill. Stigmastanol, a naturally occurring phytosterol, was found to be highly correlated to egg production. The average concentration of stigmastanol present in the concentration of effluent reducing egg production by 25% was 4.8 µg/L. Although the correlation between stigmastanol concentrations and egg production was high, only a life-cycle test with this compound could determine if stigmastanol caused egg production to be reduced at concentrations found in effluents.

The results of a fathead minnow life-cycle test with stigmastanol are reported in this technical bulletin. *Ceriodaphnia dubia* and fathead minnow 7-d tests were also completed to determine if stigmastanol affects organisms used in tests frequently required in NPDES permits, at concentrations found in effluents.

During *Ceriodaphnia* and fathead minnow 7-d tests, stigmastanol concentrations of >99 µg/L (highest concentration tested) did not alter the survival or reproduction of *Ceriodaphnia* or the survival and growth of fathead minnows. Survival and growth of fathead minnow larvae were not altered by the highest concentrations of stigmastanol tested. Stigmastanol exposures during the entire life-cycle test, including the spawning period, did not alter egg production, egg size, egg hatchability, adult survival, adult growth, gonad somatic indices, liver somatic indices and condition factors of the adult fathead minnows.

This phytosterol does not appear to cause any of the changes in *Ceriodaphnia* or fathead minnow reproduction found in earlier laboratory tests with whole effluents. If, after additional testing with other effluents, stigmastanol continues to be highly correlated to egg production, this compound may be a useful surrogate to predict concentrations of other biologically active components.

This technical bulletin is one in a series of reports already published, or now in production, that describe NCASI's ongoing studies exposing important test organisms to biologically treated mill effluents or effluent components and wood leachates. NCASI will continue to report observations of effects on survival, growth and reproduction, assessment of biomarker responses, and examination of the relationships of biomarker responses and effluent chemical components to reproduction rates. The latest studies in this series are designed to incorporate concepts developed at a North American workshop on fish reproduction research strategies. Sponsored by NCASI and AF&PA in 1997, the workshop was attended by representatives from industry, government, and academia.

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September 1999

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ABSTRACT

Previous NCASI laboratory studies found that effluent from kraft mills, bleached kraft mills, and an OCC recycle mill reduce egg production during fathead minnow life-cycle tests at concentrations from 18% to 100% v/v of effluent. Although the concentrations causing effects were much greater than most in-stream water concentrations, determination of the source of these effects may be useful because, as processes are modernized, identified sources may be considered for reduction. Two of the goals of the aquatic biology studies—of which the work reported herein is a component—are to: (1) determine if specific compounds in the effluents alter fish reproduction, and (2) determine the sources of compounds that affect reproduction. Comparisons between concentrations of components measured in pulp and paper mill effluents and reproduction endpoints from fathead minnow life-cycle tests found a very limited number of strong correlations. The strongest correlation was with stigmastanol, a naturally occurring phytosterol. Therefore, this compound was tested to determine if it may have been responsible for effects on reproduction during previous life-cycle bioassays. Fathead minnow (*Pimephales promelas*) 7-d, 28-d, 56-d, and life-cycle bioassays, as well as several biomarker responses, and a *Ceriodaphnia dubia* 7-d bioassay were completed using stigmastanol, dissolved in an acetone carrier. *C. dubia* 7-d survival and reproduction were not reduced from the controls in any of the stigmastanol concentrations (NOEC=99.2 µg/L), and the IC25s were >99.2 µg/L of stigmastanol, which was the highest concentration tested. Survival and growth of fathead minnows during one 7-d bioassay were reduced at the highest stigmastanol concentration tested (99.2 µg/L) and the acetone carrier; therefore, the responses were probably due to the acetone and not stigmastanol.

One fathead minnow 28-d survival and growth test using eggs from laboratory cultures found no effects on egg hatchability, larval or juvenile survival, or juvenile growth when exposed to stigmastanol concentrations averaging 98.4(±64.5) µg/L, the highest concentration tested. Survival and growth of juvenile fathead minnows at 56-days were not significantly different from the controls in any of the stigmastanol concentrations, and the IC25s were >110.9(±83.2) µg/L of stigmastanol. During a 28-d fathead minnow survival and growth test, using eggs spawned by fish exposed in a life-cycle test, egg hatchability, juvenile survival and juvenile growth were not adversely affected by an average stigmastanol concentration of 16(±12.2) µg/L, the highest concentration tested.

Fathead minnow life-cycle egg production parameters (total eggs, total spawns, and eggs/female/day) were not significantly different from the control egg production in any of the stigmastanol concentrations and all IC25s were >72.5±70.3 µg/L of stigmastanol, the highest concentration tested. Hatchability of the eggs produced during life-cycle testing was not altered from the control at the highest concentration, and the IC25 was >72.5±70.3 µg/L of stigmastanol. Fathead minnow egg sizes were very uniform, and differences of only 2% between groups were detected as significant. The very small differences detected as significant, and the variability between replicates within a concentration, indicate that changes observed were not due to stigmastanol. No significant differences from the control were detected for either male or female fathead minnow GSIs during

life-cycle testing. The LSIs of male fathead minnows were also not altered from the controls. Condition factors (K) of male and female fathead minnows from all stigmastanol concentrations during life-cycle testing were not significantly different from the Ks of the same sex from the controls, and the IC25s for both male and female Ks were >72.5(±70.3) µg/L of stigmastanol. Stigmastanol had no effects on any measured endpoint at concentrations ranging from 3- to over 20-times greater than the average concentration of 4.8 µg/L calculated to be in the IC25 concentrations of effluent from the six previous life-cycle tests. Thus, stigmastanol is probably not a causative factor in the effects of the pulp and paper mill effluent on fish reproduction; however, the high correlation previously observed indicates that this compound may be a surrogate for other biologically active compounds originating in the pulping process.

KEYWORDS

phytosterol, stigmastanol, fathead minnow, *Ceriodaphnia dubia*, life-cycle, reproduction, biomarkers

RELATED NCASI PUBLICATIONS

Technical Bulletin No. 755 (May 1998). *Effects of biologically treated unbleached kraft pulp mill effluent on early life-stages and life-cycles of fathead minnow* (*Pimephales promelas) and Ceriodaphnia dubia.*

Technical Bulletin No. 746 (October 1997). *Development and evaluation of a method for the determination of phytosterols in pulp and paper mill effluents.*

Technical Bulletin No. 732 (March 1997). *Effects of biologically treated elemental chlorine free bleached kraft mill effluent on early life-stages and life-cycles of the fathead minnow (Pimephales promelas) and Ceriodaphnia dubia.*

Technical Bulletin No. 722 (August 1996). *Effects of biologically treated bleached kraft mill effluent on the early life-stage and life-cycle of Pimephales promelas (fathead minnow) and Ceriodaphnia dubia: A comparison before and after conversion to oxygen delignification and ECF bleaching.*

Technical Bulletin No. 475 (December 1985). *Effects of biologically treated bleached kraft mill effluent during early life-stage and full life-cycle studies with fish.*

CONTENTS

TABLES

EFFECTS OF THE PHYTOSTEROL STIGMASTANOL ON EARLY LIFE-STAGES AND LIFE-CYCLES OF FATHEAD MINNOWS (*Pimephales promelas*) AND *Ceriodaphnia dubia*

1.0 INTRODUCTION

Concerns about the compatibility of pulp mill effluents with the integrity of aquatic communities have recently focused on fish reproduction effects (NCASI 1985, 1996, 1997a, 1998, in preparation a, b, c; Kovacs et al. 1995a, 1995b; Kovacs and Gibbons et al. 1996; Kovacs and Voss et al. 1996; Robinson 1994; Owens 1991; Adams et al. 1992; Bortone and Davis 1994; Munkittrick et al. 1991, 1992, 1994; McMaster et al. 1991; Förlin et al. 1991; Sandström 1994). Some investigations have suggested that phytosterols found in pulp mill effluents may cause some or all of the effects on fish reproduction (Rosa-Molinar and Williams 1984; Denton et al. 1985; Hunsinger et al. 1988; MacLatchy and Van Der Kraak 1995; Mellanen et al. 1996; Knutson et al. 1997). Phytosterol levels in European kraft mill effluent have been reported frequently (Holmbom 1980; Holmbom and Lehtinen 1980; Dahlman et al. 1993; Oikari and Holmbom 1996; Strömberg et al. 1996) and a few studies with thermomechanical (TMP) pulp mill effluent (Ekman and Holmbom 1989; Ekman et al. 1990; Carlberg 1993) are reported. The levels of phytosterols in U.S. pulp and paper mill effluents of various types are also available (NCASI 1997b, NCASI unpublished). Preliminary screening of 22 pulp and paper mill effluents in the United States indicated that β-sitosterol, stigmasterol, campesterol, and stigmastanol were the most common phytosterols present (NCASI 1997b). Chemical characteristics of several types of whole pulp and paper mill effluents in the U.S. have been studied by NCASI during life-cycle testing on fish reproduction effects (NCASI 1985, 1993, 1996, 1997a, 1998, in preparation a, b, c). Previous studies found that effluent from kraft mills, bleached kraft mills, and an OCC recycle mill reduced egg production during fathead minnow life-cycle tests at concentrations from 18% to 100% v/v of effluent. Although these effect levels are high compared to in-stream water concentrations, determination of the source of these effects may be useful because as modernization of processes occur these sources may be considered for reduction. Linear regression analyses of the relationships between many of the effluent components and IC25s for fish reproduction from fathead minnow life-cycles with effluents from four bleached kraft mills, one unbleached kraft mill and one OCC recycled fiber mill, found a significant correlation (r^2=0.76; $p<0.03$) with stigmastanol, one of the four major phytosterols found in U.S. mill effluents (NCASI 1997b, NCASI unpublished).

The primary objective of the research reported in this bulletin was to determine if the concentrations of stigmastanol found in U.S. pulp and paper mill effluents could be a cause of reduced egg production found in previous studies (NCASI 1985, 1993, 1996, 1997a, 1998, in preparation a, b, c). In addition to the life-cycle studies, 7-d bioassays using fathead minnows and *C. dubia* were also performed to determine if this compound could cause adverse effects in tests required in many U.S. NPDES permits.

This technical bulletin presents the results of one *Ceriodaphnia dubia* 7-d survival and reproduction bioassay and one fathead minnow 7-d survival and growth bioassay with stigmastanol. The results of a 28-d fathead minnow early life-stage survival and growth test using eggs from cultures in well water, and a 28-d test using eggs from fish exposed to stigmastanol during life-cycle tests are reported. The results of a 56-d fathead minnow growth and survival test (a continuation of the first 28-d test using eggs from laboratory cultures) and a fathead minnow life-cycle test for survival, growth, egg production, and egg hatchability, are also reported in this technical bulletin.

Some researchers have suggested that effluent effects on physiological and biochemical indices ("biomarkers") in fish can be used as indicators of reproductive fitness (Robinson 1994; Adams et al. 1992; Munkittrick et al. 1991, 1992, 1994; McMaster et al. 1991; Förlin et al. 1991; Sandström 1994). Several such physiological indices were measured during this life-cycle test and the results are included in this report. These measurements include egg size, gonad somatic indices (GSIs), liver somatic indices (LSIs) and condition factors (Ks).

2.0 TEST FACILITY AND EXPERIMENTAL METHODS

2.1 Facility Description and Dilution Water

All testing described in this bulletin was performed at the NCASI Southeastern Aquatic Biology Facility in New Bern, North Carolina. The temperature of the laboratory was maintained at 25±2°C at all times. Dissolved oxygen in exposure tanks was maintained above 7 mg/L by aeration. Lighting was controlled inside the lab between 90 and 100 ft-c by banks of fluorescent lights equipped with broad-spectrum bulbs (Durotest Vitalight). An electrical timer controlled the photoperiod to 16 hrs of light and 8 hrs of darkness over 24 hrs. Cultures of both *C. dubia* and fathead minnows were maintained in this laboratory and provided the organisms for all of the tests described in this bulletin.

The dilution water for fathead minnow egg hatchability, 28-d, 56-d and life-cycle tests was from a 50 m deep well located at the site. Characteristics of the well water are shown in Table 1. The well water was pumped to a 6200-L polyethylene holding tank equipped with a float valve, which allowed new water to enter as it was used. Well water was pumped from the holding tank to a fiberglass tank inside the laboratory, where the water was aerated and heated to the desired test temperature. A float switch in the tank allowed water to flow into the tank as needed. Diluent was pumped to two solenoid proportional diluters (USEPA 1978) through CPVC pipe connected to a centrifugal pump.

Table 1. Summary of Water Quality Characteristics of the Dilution Water During a Fathead Minnow Life-Cycle Test with Stigmastanol

Parameter	N[a]	Units	Average Concentration	(SD)[b]
pH	11	units	8.2	(0.1)
Conductivity	12	µmhos/cm	362	(8.1)
Hardness	11	mg/L	165	(9.4)
Alkalinity	11	mg/L	188	(4.7)

[a] number of samples
[b] standard deviation

Dilution water for the *C. dubia* and fathead minnow 7-d bioassays was synthetic, EPA moderately hard water (USEPA 1989). Well water at the laboratory was charcoal filtered, passed through two deionizing tanks, a Millipore Milli-Q system and a Barnstead D8904 organic removal cartridge before being reconstituted to the moderately hard diluent. Water quality parameters during testing were as follows: hardness 82(±2) mg/L, alkalinity 62(±2) mg/L, pH 7.8(±0.1) and conductivity averaged 290(±2) µmhos/cm (APHA 1992).

2.2 Stigmastanol Quality Assurance and Concentrations

A stigmastanol standard purchased from Sigma Chemical Company was found to be 98.2% pure (NCASI 1997b). A concentrated stigmastanol solution was prepared by adding 800 mg of the stigmastanol standard to 220 ml of GC^2 acetone; the minimum amount of acetone needed to dissolve the stigmastanol at ambient temperature. The concentrated stigmastanol solution was metered into a fiberglass holding tank containing well water to a nominal test concentration of 200 µg/L stigmastanol and 0.0055% v/v acetone. The test solution was temperature adjusted to 25°C. This concentration based on pretest studies that indicated as much as 75% of the stigmastanol would be removed from the water column when actual exposure tank concentrations were measured. Since the average concentration of stigmastanol in the IC25 concentration for egg production was 4.8 µg/L, this was expected to provide a high concentration at least 10-times greater than the average concentrations found at the effluent IC25s during earlier studies. A grab sample was collected from the life-cycle exposure tanks weekly and analyzed for concentrations of phytosterols (NCASI 1997b). A centrifugal pump distributed the stigmastanol test solution to the two diluter systems through polyethylene tubing.

A GC^2 acetone carrier (blank) testing solution was made by adding 220 mls of acetone to 4000 Ls of well water in a 6200-L polyethylene holding tank. A final acetone concentration of 0.0055% v/v was achieved. New blank solution was made each week during the life-cycle study. The 6200-L holding tank supplied a 113-L polyethylene tank inside the laboratory where the solution was aerated and temperature adjusted to 25°C. A centrifugal pump distributed the blank solution to the two diluter systems through polyethylene tubing.

2.3 Chronology of the Investigations

A detailed chronology of the bioassays performed is provided in Table 2. Experimental methods for the exposures with this effluent are summarized in the following sections.

Table 2. Chronology of the Investigations

Date	Test	Comment
Sept. 97	Fathead minnow 28-d growth and survival	Eggs from well water
Oct. 97	Fathead minnow 56-d growth and survival	Continued from 28-d
Sept.97-Dec. 97	Fathead minnow life-cycle	Continued from 28-d and 56-d bioassays
	Egg production	
	Egg hatchability	
	Egg size	
	Adult survival	
	Adult weight and length	
	Condition factor	
	Gonad somatic index	
	Liver somatic index	
Dec. 97	Fathead minnow 28-d growth and survival	Eggs from exposed life-cycle fish
Mar. 98	*C. dubia* 7-d reproduction and survival	
	Fathead minnow 7-d growth and survival	Eggs from well water

2.4 *Ceriodaphnia dubia* 7-d Survival and Reproduction Bioassay

A *C. dubia* life-cycle test was initiated with neonates <24 hr old, and produced within an 8-hr time period from ten randomly selected pretest culture organisms. Diluent and culture media were EPA moderately hard water as described in section 2.1. The minimum pretest reproduction criterion was a mean of 20 young/adult for 3 broods or 7 days, whichever came first. Chronic testing followed procedures described in USEPA/600/4-89/001 (1989). Standard methods and deviations from EPA methods during this test are summarized in Table 3.

Table 3. Summary of *C. dubia* 7-Day Bioassay Conditions

	Parameter	Test Condition
1.	Temperature	25°C±1°C
2.	Photoperiod	16 hr light/8 hr dark
3.	Test vessel	30-ml glass beaker
4.	Volume of test solution	15 ml
5.	Renewal	Daily
6.	Age at start	8-hr age span, <24 hr old
7.	Number/beaker	1
8.	Replicates/concentration	10
9.	Food[a]	0.5 ml/15 ml test solution
10.	Food mixture[b]	1 part *Selenastrum capricornutum* concentrate (62 x 10^6 cells/ml) 2 parts YTC (yeast, fermented trout chow, cerophyll) 4 parts extra ferm. trout chow
11.	Diluent	EPA moderately hard
12.	End points	7 days or 3 broods Control 80% survival 20 young/adult

[a] deviation from standard methods
[b] YTC and *Selenastrum* concentrate were mixed before feeding

Neonates were individually placed in 30-ml glass beakers with 15 ml of test solution and 0.5 ml of food mixture in a random block design. All beakers were placed in a styrofoam box with a glass top to limit temperature variation and evaporation. Temperature was maintained at 25±1°C inside an environmental chamber. Original individuals were moved into clean test beakers containing new media and food each day when the survival and number of young produced were recorded. Water chemistry was monitored when the organisms were moved to new solutions in accordance with Table 4. Test criteria required that at least 80% of the controls survived and at least 20 young/adult were produced.

Table 4. Summary of Water Quality Measurements During *C. dubia* 7-Day Bioassay

Parameter	Day	Test Solution	Test Concentration
Hardness	0	New	Control / Blank / Highest
	6	New	Control / Blank / Highest[a]
Alkalinity	0	New	Control / Blank / Highest
	6	New	Control / Blank / Highest[a]
Temperature	0-6	New	Control / Blank + all concentrations
	1-7	Old	Four corners and middle of test chamber
Conductivity	0-6	New	Control / Blank + all concentrations
pH	0,3,6	New	Control / Blank, Low, Middle, High
	1,4,7	Old	Control / Blank, Low, Middle, High
Dissolved Oxygen	0,3,6	New	Control / Blank, Low, Middle, High
	1,4,7	Old	Control / Blank, Low, Middle, High

[a] highest concentration with survivors

2.5 Fathead Minnow 7-d Survival and Growth Bioassay

A fathead minnow 7-d survival and growth bioassay was initiated with larvae <24 hr old produced during a 12-hr time span, from at least four different spawns. EPA moderately hard water as described in section 2.1 was used as the diluent during the testing. Chronic testing followed procedures outlined in USEPA/600/4-89/001 (1989). Standard methods and deviations from the EPA methods during this test are listed in Table 5.

Table 5. Summary of Fathead Minnow 7-Day Bioassay Conditions

	Parameter	Test Conditions
1.	Temperature	25 ± 1°C
2.	Photoperiod	16 hr light/8 hr dark
3.	Light intensity	80-100 footcandles
4.	Test vessel	500-ml glass beaker
5.	Test volume	250 ml
6.	Renewal	Daily
7.	Age at start	<24 hr/12 hr age span
8.	Number per beaker	10
9.	Number replicates	4
10.	Food[a]	0.5 ml of a 1600/ml *Artemia* suspension 3 times/day increased to 1.0 ml on days 3-6
11.	Diluent	EPA moderately hard

[a] deviation from standard methods

Ten larvae were individually placed in 500-ml glass beakers with 250 ml of test solution and food. Four replicate beakers/concentration were placed in a heated water bath at 25±1°C in a random block design. Each beaker was fed 0.5 ml of a 1600/ml suspension of <24hr old *Artemia franciscana* nauplii three times daily for the first two days. The food was increased to 1.0 ml on days 3 through 6. All beakers were covered with a watch glass to prevent evaporation and airborne contaminants. The beakers were cleaned and exchanged daily with new test solution and food. At that time, survival was recorded and water chemistry monitored as shown in Table 6. On day seven the fish were not fed. The larvae were dried at 100°C for at least 2 hr and then weighed to the nearest 0.1 mg. Survival in the four control replicates averaged at least 80% and the weights averaged at least 0.25 mg/fish or the test was not acceptable.

Table 6. Summary of Water Quality Measurements During a Fathead Minnow 7-Day Bioassay

Parameter	Day	Test Solution	Test Concentration
Hardness	0	New	Control / Blank + Highest
	6	New	Control / Blank + Highest[a]
Alkalinity	0	New	Control / Blank + Highest
	6	New	Control / Blank + Highest[a]
Temperature	0-6	New	Control / Blank + All concentrations
	1-7	Old	Control / Blank + All concentrations
Conductivity	0-6	New	Control / Blank + All concentrations
	1-7	Old	Control / Blank + All concentrations
pH	0-6	New	Control / Blank + All concentrations
	1-7	Old	Control / Blank + All concentrations
Dissolved	0-6	New	Control / Blank + All concentrations
Oxygen	1-7	Old	Control / Blank + All concentrations

[a] highest concentration with survivors

2.6 Fathead Minnow 28-d and 56-d Survival and Growth Bioassays

One fathead minnow early life-stage 28-d survival and growth bioassay and one 56-d bioassay were performed in exposure system #1 (es1) during the life-cycle exposures. The 56-d bioassay was a continuation of the first 28-d test at the start of the life-cycle. All of these tests began with eggs from laboratory well-water cultures. One 28-d test completed during life-cycle testing began with eggs from the exposed fish (Table 2). All procedures followed those of the first 28-d, and 56-d life-cycle test periods as described in section 2.7.

2.7 Fathead Minnow Life-Cycle Test

A fathead minnow life-cycle test was completed using two test systems, each with a proportional solenoid-valve diluter (USEPA 1978), and aquaria to hold the fish in the test concentrations. The diluters were modified with a solenoid switch to discharge the entire volume of the test solutions alternately into the A or B replicate test chambers. Flow rates to each test chamber averaged approximately 1000 ml/5 min. cycle. Replicate A and B test chambers were randomly selected for exposure to the stigmastanol concentrations. The light intensity at the water surface of each chamber was maintained at 100-130 ft-c using broad-spectrum (Vitalite) fluorescent lights mounted over the tanks. Dissolved oxygen in each chamber was maintained above 7 mg/L using an oil-free blower and

airstones. The initial exposure system (es1) consisted of 14 tanks, 33 cm x 33 cm x 16 cm deep, which allowed two replicates for each of six concentrations and an acetone blank. A self starting siphon in each chamber controlled the solution depth between 7 and 12 cm. This diluter was used to start the life-cycle bioassay and complete all egg hatchability, 28-d and 56-d fathead minnow tests.

The fathead minnow life-cycle test was initiated when eggs spawned in laboratory cultures were placed in 25-cm x 8-cm x 13-cm deep hatching chambers, divided by glass partitions into four equal 6-cm subchambers. Stainless steel screen mesh (0.0145", 50 mesh/inch) was attached to the bottom of the chambers with silicone sealant. The chambers were suspended with 1-cm dowels attached to the sides with silicone sealant, which rested on the top edge of the exposure tanks of es1. Twenty-five eggs were placed in each of the 6-cm subchambers, for a total of 100 eggs per hatching chamber with two replicates per concentration. The siphon drain system of es1 caused the tank fluid levels to fluctuate between 7-12 cm, which in turn exchanged the test solutions in the hatching chambers. Unfertile and dead eggs were removed twice daily. Hatching occurred by day 4 or 5, when the live larvae were counted and the percent hatch was calculated.

Fifty of the larvae <24 hr old were pipetted into 26-cm x 17-cm x 13-cm deep glass growout chambers which were placed in the exposure tanks of es1. Stainless steel wire mesh (0.0145", 50 mesh/inch) covered the bottom of each growout tank. The growout chambers were suspended off the bottom of the test tanks for circulation. The self-starting-siphon system exchanged test solutions inside the growout chambers 8-10 times daily. The larvae were fed newly hatched (<24hr old) *Artemia* nauplii 3 times a day at the initial rate of 3 ml/chamber of a 1600/ml suspension. This amount was increased on a weekly basis to a maximum of 12 ml by day 23. All tanks and chambers were monitored twice daily, and cleaned every 7 days to remove extra food and waste. On day 28 the fish were not fed. Larvae were individually weighed on day 28 to the nearest 0.1 mg in a tared 60-ml beaker of water, and returned to clean growout chambers to continue the life-cycle testing. The same procedures as outlined for the first 28 days were used from day 28 through day 56. The food was supplemented with 2 ml of a 250 g/L frozen brine shrimp slurry on day 29, and increased to 5 ml by day 55. On day 56 the fish were blotted dry, weighed alive to the nearest 0.1 mg, and moved to a second exposure system (es2) for the reproduction portion of the life-cycle test.

Es2 was similar to es1 except the chambers were replaced by 61-cm x 31-cm x 31-cm deep glass aquaria. Each aquarium had a drain, which maintained the solution depth at 19 cm. Initially the aquaria were divided into 2 equal 30-cm x 30-cm compartments by 3-mm stainless steel mesh screens. When spawning began, the aquaria were split into four, equal 30-cm x 15-cm compartments using the same screening material.

The fathead minnows were held in each concentration as two groups in each of the two replicates, (4 groups/concentration) until day 75-80. When males began to show spawning coloration, one male and two females were placed in each of the four spawning chambers in each aquarium. Since each concentration had replicate aquaria, eight spawning groups were exposed to each concentration. Spawning fish were fed 3 ml/chamber of the 250 g/L brine shrimp slurry twice daily. A 75-mm wide half section of 115-mm stainless-steel pipe was placed in each chamber as spawning substrate. Spawning usually started between 80-90 days after hatching. The numbers of eggs and spawns, along with mortality, were recorded daily throughout the spawning period. The spawning was stopped after six weeks and the test terminated. The adult fish were measured, weighed, gonads and livers excised, and the sex confirmed.

Spawns were collected for egg hatchability from as many different spawning chambers as possible throughout the reproduction period. The hatching chambers described earlier in this section held the eggs in es1 during hatchability tests. Twenty-five eggs were placed in each of the four subchambers

for a total of 100 eggs/spawn. The eggs were checked twice daily for viability and dead eggs were removed. The larvae were counted after the eggs hatched on day 4 or 5, and the percent hatch was computed.

2.8 Biomarkers

2.8.1 *Egg size*

Eggs were collected every week at the same time of day ± 2 hr over the reproduction period and measured with an ocular micrometer. Fifty eggs from six different spawns in each concentration were measured during the fathead minnow life-cycle reproduction period. All egg measurements from each concentration were pooled for statistical evaluations.

2.8.2 *GSIs*

The gonads from male and female fathead minnows were excised and weighed at the termination of the life-cycle test. The weight of the gonad was divided by the weight of the fish and the result was multiplied by 100 to calculate GSIs reported in this bulletin (Bagenal 1978).

2.8.3 *LSIs*

Livers of male fathead minnows from each test concentration were collected at the termination of the life-cycle test. LSIs reported in this bulletin were calculated by dividing the weight of the liver by the total weight of the fish and multiplying the results by 100 (Bagenal 1978).

2.8.4 *Condition Factor*

The condition factor (K) was calculated for each test concentration at the termination of the life-cycle test on the male and female fathead minnows using the formula:

$$K=(100)(w)/L^3$$

where w is the weight in milligrams and L is the total length of the fish in millimeters (Bagenal 1978).

2.9 Statistical Evaluations

Biological data were subjected to either parametric or nonparametric statistical analyses. The analyses were selected based upon whether the data met the assumptions for parametric testing using the Kolmogorov Test for normality from the VARF statistical program (Shapiro and Francia 1971; Stephens 1974) and Bartlett's Test for homogeneity of variance from the Toxstat statistical program (Gulley 1988). The $p \leq 0.05$ level was used as an indication of significant differences unless otherwise noted. Usually Dunnett's (parametric) or Steel's (nonparametric) test was used for multiple comparisons (Steel and Torrie 1980; Rodgers 1986; NWA 1984). The Bootstrp regression model program (Efron 1982) was used to estimate the concentration producing a 25% reduction in the response means (IC25) and 95% confidence intervals. The IC25 from the linear interpolation model is reported.

C. dubia 7-d test data were analyzed for differences in survival among groups using Fisher's Exact Test and the reproduction data were analyzed using both Dunnett's (parametric) and Steel's (nonparametric) tests (Rodgers 1986). The Bootstrp program was used to calculate the IC25 and 95% confidence interval for both survival and reproduction.

Fathead minnow 7-d test survival data were arc-sine transformed before statistical analysis using Fisher's Exact Test (NWA 1984). The final weights were analyzed using Dunnett's Test and Steel's

Test for differences from the control (Rodgers 1986). The Bootstrp program was used to calculate the IC25 and the 95% confidence intervals for both survival and growth (Efron 1982).

Analysis of the results of the fathead minnow 28-d and 56-d survival and growth tests indicated a normal distribution of the average weights in a majority of the test results; thus, analysis of variance (ANOV) and Dunnett's Test were used for the analyses (NWA 1984). Fisher's Exact Test was used to determine significant differences for survival. The Bootstrp program was used to calculate the IC25 and 95% confidence intervals for both survival and average weights (Efron 1982). Student's t-Test was used to determine significance of differences in average weights between the two A and B replicates (NWA 1984).

The assumptions for parametric testing were not met for several egg production parameters; thus, Steel's Many One Rank Test was used for comparisons of total eggs, total spawns, and eggs/female/day (e/f/d) (Gulley et al. 1988). Fisher's Exact Test was used to determine significant differences for survival of the adult fish. The Kruskal-Wallis Test was used to detect differences in final weights for both the male and female fathead minnows (NWA 1984). The IC25 and 95% confidence intervals were calculated for each parameter using the Bootstrp program (Efron 1982).

Biomarker data for egg size were subjected to ANOV and Dunnett's Test, having met the criteria for parametric testing (NWA 1984). The GSI, LSI, and K data did not meet criteria for parametric testing; thus, Steel's Many One Rank Test or the Kruskal-Wallis Test was used for comparisons (Gulley 1988; NWA 1984). The IC25 was calculated for each parameter using the Bootstrp computer program (Efron 1982).

3.0 RESULTS

3.1 Stigmastanol Concentrations

Average stigmastanol concentrations as detected by GC/MS procedures (NCASI 1997b) from samples collected from the exposure tanks during fathead minnow life-cycle testing are presented in Table 7. The concentrations of four major phytosterols found in US pulp and paper mill effluents for each sample collected from the exposure tanks are provided in Appendix A and the average concentrations of the phytosterols during each test period are provided in Appendix B. The amount of stigmastanol detected from samples collected during life-cycle testing was reduced by over 60% from the initial concentration added to the mixing tank (200 µg/L).

Table 7. Average Stigmastanol Concentrations Measured in Samples Taken from Exposure Tanks During Fathead Minnow Life-Cycle Testing

Parameter	Control	Blank	Stigmastanol Concentrations (µg/L)				
Average[a]	0.0	0.0	1.3	2.5	8.8	26.2	72.5
(SD)[b]	(0)	(0)	(1.4)	(2.5)	(9.3)	(27.9)	(70.3)

[a] average of 14-17 samples
[b] standard deviation

3.2 *Ceriodaphnia dubia* 7-d Life-Cycle Bioassay Results

Survival of *C. dubia* in all stigmastanol concentrations and the acetone carrier was not significantly different from survival of the controls; thus, the NOEC for survival was 99.2 µg/L and the IC25 was >99.2 µg/L (Table 8). Reproduction was also not significantly altered from the control at any of the stigmastanol concentrations and the IC25 was >99.2 µg/L of stigmastanol (highest concentration tested).

Table 8. Results of a *C. dubia* 7-Day Bioassay with Stigmastanol

Date	Parameter	Control	Blank	Stigmastanol Concentration (µg/L)					IC25
	Concentration	0.0	0.0	ND[a]	2.0	11.7	35.7	99.2	
03/19/98 to 03/25/98	Survival Average (%)	100	100	100	100	100	100	100	>99.2
	Reproduction Avg. No./Adult[b]	33.0	32.9	33.4	30.0	31.5	31.0	32.1	>99.2
	SD[c]	3.2	3.2	2.5	5.7	2.9	5.5	2.4	
Survival	NOEC = 99.2 µg/L			IC25 = >99.2 µg/L					
Reproduction	NOEC = 99.2 µg/L			IC25 = >99.2 µg/L					

[a] below detection limit (0.44 µg/L)
[b] average number of young produced per adult
[c] standard deviation

3.3 Fathead Minnow 7-d Bioassay Results

Survival of fathead minnows during one 7-d bioassay was significantly reduced from the controls at the highest stigmastanol concentration tested, 99.2 µg/L and in the acetone carrier (NOEC = 35.7 µg/L). The IC25 for survival was 97.2 µg/L of stigmastanol (Table 9). Average weights of the larvae on day seven were also significantly reduced at the highest stigmastanol concentration and the acetone carrier. The IC25 for average weights was 95.8 µg/L stigmastanol. Since the survival and average weights were reduced in both the control blank and the highest stigmastanol concentration, which have equal amounts of the acetone carrier, this effect was probably due to the carrier and not from exposure to stigmastanol, or other factors may have caused mortality in both chambers.

Table 9. Results of a Fathead Minnow 7-Day Bioassay With Stigmastanol

Date	Parameter	Control	Blank	Exposure Tank Stigmastanol Concentration (µg/L)					IC25
	Concentration	0.0	0.0	ND[a]	2.0	11.7	35.7	99.2	
03/19/98 to 03/25/98	Survival Average %	88	68f[b]	85	83	85	85	63f	97.2
	Growth Avg. Wgt.(mg)[c]	10.3	7.8*[d]	9.1	9.0	10.4	10.4	7.4*	95.8
	SD[e]	1.1	1.4	1.1	1.0	1.4	1.2	2.9	
Survival		NOEC = 35.7 µg/L		IC25 = 97.2 µg/L					
Growth		NOEC = 35.7 µg/L		IC25 = 95.8 µg/L					

[a] below detection limit (0.44 µg/L)
[b] significant Fisher's Test (p≤0.05)
[c] average weight
[d] significant Dunnett's Test (p≤0.05)
[e] standard deviation

3.4 Fathead Minnow 28-d and 56-d Early Life-Stage Results

The results of one 28-d fathead minnow survival and growth test using eggs spawned in well water and exposed to stigmastanol and an acetone blank are presented in Table 10. Responses from replicate A and replicate B were rarely significantly different from each other; thus, they were combined for analyses of stigmastanol effects. The hatchability of eggs spawned in well water and exposed to stigmastanol during the start of the 28-d test was not significantly reduced in any stigmastanol concentration. The IC25 for egg hatchability was >98.4(±64.5) µg/L of stigmastanol (highest concentration tested). Juvenile survival was not significantly altered from the control in any of the stigmastanol concentrations during the 28-d test and the IC25 was >98.4(±64.5) µg/L. Average weight (growth) of the 28-d old fathead minnows was significantly greater than the control weight at the 31.6(±24.7) µg/L concentration of stigmastanol. Average weight of the larvae from the highest stigmastanol concentration tested (98.4(±64.5) µg/L) was not significantly altered from the control. The lack of a consistent effect on growth by the stigmastanol and the observation that the higher concentration did not have an effect indicates the response at 31.6(±24.7) µg/L was probably not stigmastanol induced. The IC25 for average weight was >98.4(±64.5) µg/L of stigmastanol.

Survival of fathead minnow larvae at 56 days was not significantly different from the control in any of the stigmastanol concentrations and the IC25 was >110.9(±83.2) µg/L of stigmastanol (Table 11). The average weights of fathead minnows at 56 days were also not significantly different (p≤0.05) from the control and the IC25 was >110.9(±83.2) µg/L.

Table 10. Fathead Minnow 28-Day Early Life-Stage Results Using Eggs Spawned in Well Water and Exposed to Stigmastanol

Date	Parameter	Control	Blank	Exposure Tank Stigmastanol Concentration (µg/L)					IC25
	Average[a]	0.0	0.0	1.2	4.3	13.3	31.6	98.4	
	(SD)[b]	(0)	(0)	(0.2)	(1.0)	(6.1)	(24.7)	(64.5)	
08/21/97	Hatch (%)	90	93	85	88	84	89	92	>98.4
09/18/97	28-d Sur.(%)[d]	94	94	94	94	93	90	97	>98.4
	28-d Average weight (mg)	99.6	103.8	101.9	93.9	103.3	109.7*[e]	96.3	>98.4
	(SD)	(26)	(23)	(20)	(25)	(21)	(23)	(23)	

[a] average of 4 samples
[b] standard deviation
[c] significant Fisher's Test (p≤0.05)
[d] percent survival
[e] significant Dunnett's Test (p≤0.05)

Table 11. Fathead Minnow 56-Day Early Life-Stage Results Using Eggs Spawned in Well Water and Exposed to Stigmastanol

Date	Parameter	Control	Blank	Exposure Tank Stigmastanol Concentration (µg/L)					IC25
	Average[a]	0.0	0.0	1.8	4.1	14.3	40.2	110.9	
	(SD)[b]	(0)	(0)	(1.5)	(2.5)	(10.0)	(34.8)	(83.2)	
08/21/97 10/16/97	56-d Sur.(%)[c]	89	92	92	90	91	87	92	>110.9
	56-d Average weight (mg)	287.8	282.1	284.5	263.6	280.5	296.6	283.4	>110.9
	(SD)	(71)	(78)	(63)	(63)	(64)	(69)	(59)	

[a] average of 8 samples
[b] standard deviation
[c] percent survival

The results of one fathead minnow 28-d growth and survival test using eggs spawned by exposed fish during life-cycle testing are presented in Table 12. Egg hatchability during the test was significantly reduced from the control in the acetone carrier, the 0.6(±0.4) µg/L test concentration and in the 5.7(±4.3) µg/L stigmastanol concentration. Hatchability of eggs at the highest stigmastanol concentration tested (16(±12) µg/L) was not altered from the control, therefore the responses observed at the lower concentrations were probably not stigmastanol related and probably reflected the variability of different groups of eggs. However, the acetone may have played some role because eggs from the blank and all concentrations were less than the control during the life-cycle study (Table 13) and generally poorer than in other studies (NCASI 1985, 1996, 1997a, 1998, in preparation a, b, c). The IC25 for egg hatchability during this test with eggs from exposed life-cycle fish was >16(±12) µg/L stigmastanol (highest concentration tested). Survival of juvenile fish at

28-days was significantly reduced from the control in the acetone carrier and at the 0.6(±0.4) µg/L stigmastanol concentration. Survival at all higher concentrations of stigmastanol was not altered; thus, the response at the lower concentration was probably not stigmastanol related. The IC25 for survival was >16(±12) µg/L of stigmastanol. Average weights (growth) of the larval fish during the test were significantly greater than the control average weight only in the concentrations with reduced survival and the IC25 was >16(±12) µg/L stigmastanol (highest concentration tested) (Table 12). The higher growth in concentrations with reduced survival may have been due to reduced competition for food or space.

Table 12. Fathead Minnow 28-Day Early Life-Stage Results with Stigmastanol Using Eggs from Life-Cycle Exposed Fish

Date	Parameter	Control	Blank	Exposure Tank Stigmastanol Concentration (µg/L)					IC25
	Average[a]	0.0	0.0	0.2	0.6	1.3	5.6	16.0	
	(SD)[b]	(0)	(0)	(0.2)	(0.4)	(0.6)	(4.3)	(12.2)	
11/26/97	Hatch (%)	82	57f[c]	78	53f	90	70f	83	>16.6
12/23/97	28-d Sur.(%)[d]	89	74f	84	70f	83	88	87	>16.6
	28-d Average weight (mg)	98.5	133.2*[e]	109.0	115.3*	100.4	106.5	105.5	>16.0
	(SD)[d]	(21)	(20)	(30)	(31)	(27)	(29)	(26)	

[a] average of 2-3 samples
[b] standard deviation
[c] significant Fisher's Test (p≤0.05)
[d] percent survival
[e] significant Dunnett's Test (p≤0.05)

3.5 Fathead Minnow Life-Cycle Results

The average numbers of eggs produced during fathead minnow life-cycle testing in all stigmastanol concentrations were not significantly different from the control, and the IC25 was >72.6(±70.3) µg/L of stigmastanol (Table 13). The average number of spawns produced in each stigmastanol concentration was also not significantly different from controls (p≤0.05) and the IC25 was >72.6(±70.3) µg/L stigmastanol. E/f/d was not significantly altered from the control in any stigmastanol concentration tested 72.6(±70.3) µg/L and the IC25 was >72.6(±70.3) µg/L of stigmastanol. Hatchability of eggs from life-cycle exposed fish was significantly reduced from the control (p≤0.05) at the 2.5(±2.5) µg/L stigmastanol concentration. Hatchability at all higher concentrations was not altered; therefore, the response was probably not related to the stigmastanol and the IC25 for egg hatchability was >72.6(±70.3) µg/L of stigmastanol (Table 13). Survival of male and female fish to the end of the spawning period was not changed in any of the stigmastanol concentrations and the IC25s were >72.6(±70.3) µg/L stigmastanol. There were significant differences between survival of male and female fish within a concentration. This was probably caused by the aggressiveness of the males during the spawning period, resulting in mortality of the female fish and has often been found in these tests at all concentrations (NCASI 1985, 1996, 1997a, 1998, in preparation a, b, c). Final weights of the male and female fish from all stigmastanol concentrations at the end of the spawning period were not significantly different from the control fish weights of the same sex, and the IC25s were >72.6(±70.3) µg/L stigmastanol.

Table 13. Egg Production, Hatchability, Adult Survival and Final Weights During a Fathead Minnow Life-Cycle Test with Stigmastanol

Parameter	Control	Blank	Stigmastanol Concentration (µg/L) Measured in Exposure Tanks					IC25
Average Stigmastanol[a]	0.0	0.0	1.3	2.5	8.8	26.2	72.5	
(SD)[b]	(0)	(0)	(1.4)	(2.5)	(9.3)	(27.9)	(70.3)	
Total Eggs (Avg/sp grp)[c]								
Replicate A	2307	2674	2989	2725	1151	2547	1896	
(SD)	(945)	(269)	(342)	(851)	(525)	(891)	(887)	
Replicate B	3297	1748	2588	2192	2483	1657	3087	
(SD)	(235)	(938)	(1018)	(858)	(1215)	(1685)	(454)	
Total (Avg/sp grp)[d]	2802	2145	2788	2497	1912	2165	2492	>72.5
(SD)	(829)	(842)	(735)	(830)	(1156)	(1253)	(912)	
Total Spawns (Avg/sp grp)[c]								
Replicate A	14	14	14	14	8	16	12	
(SD)	(3)	(2)	(5)	(5)	(3)	(2)	(3)	
Replicate B	15	10	15	12	13	8	16	
(SD)	(3)	(4)	(5)	(4)	(5)	(6)	(3)	
Total (Avg/sp grp)	14	11	14	13	11	12	14	>72.5
(SD)	(3)	(4)	(4)	(4)	(5)	(6)	(3)	
Eggs/Female/Day (Avg/sp grp)[c]								
Replicate A	28.9	31.8	35.6	32.4	13.7	30.4	22.6	
(SD)	(9)	(3)	(4)	(10)	(6)	(6)	(11)	
Replicate B	39.3	21.9	30.8	26.1	29.6	24.5	36.8	
(SD)	(3)	(12)	(12)	(10)	(14)	(20)	(5)	
Total[d] (Avg/sp grp)	34.1	26.2	33.2	29.7	22.8	27.8	29.7	>72.5
(SD)	(8)	(10)	(9)	(10)	(14)	(14)	(11)	
Hatch %								
Total (Avg)	71.8	61.8	70.8	58.8f[e]	63.3	62.8	60.6	>72.5
(SD)	(18)	(17)	(13)	(19)	(26)	(21)	(21)	
Adult Survival %[f]								
Male (% of males)	100	100	100	100	100	100	100	>72.5
Female (% of females)	88	86	100	86	100	71	100	>75.0
Total (% of total adults)	92	90	100	90	100	81	100	>75.0
Final Weight (mg)								
Male (Avg fish)	2137	2365	2182	2397	2371	2337	2221	>72.5
(SD)	(235)	(365)	(406)	(462)	(512)	(441)	(215)	
Final Weight (mg)								
Female (avg fish)	1032	1115	1109	1061	1043	1169	1021	>72.5
(SD)	(173)	(160)	(199)	(226)	(243)	(182)	(121)	

[a] average of 14-17 samples
[b] standard deviation
[c] average per spawning group
[d] average of the eight spawning groups
[e] significant Fisher's Test ($p \leq 0.05$)
[f] survival based on 8 males and 16 females in each concentration

3.6 Biomarkers

3.6.1 *Fathead Minnow Egg Size*

Egg diameters were very uniform and differences of only 2% between groups were detected as significant (Table 14). Eggs collected during life-cycle testing from fish in 9.3(±9.4) µg/L, 26.6(±28.9) µg/L, and 72.6(±70.3) µg/L stigmastanol were significantly larger than control eggs. The IC25 for egg diameter was >72.6(±70.3) µg/L stigmastanol. Egg diameters were significantly different in 6 of 7 comparisons between the two replicates, including the acetone carrier. The very small differences detected as significant and the frequent significant differences between replicates indicates the differences observed in egg size were probably not due to the stigmastanol, and are unlikely to have population level effects.

Table 14. Diameter of Eggs from Spawns During a Fathead Minnow Life-Cycle Test with Stigmastanol

Average[a] Stigmastanol Conc. (µg/L)	(SD)[b]	Replicate A N[c]	Diameter[d]	(SD)
Control	(0)	100	1.23	(0.06)
Blank	(0)	150	1.22	(0.07)
1.3	(1.4)	200	1.23	(0.07)
2.5	(2.5)	150	1.20	(0.05)
8.6	(9.2)	100	1.23	(0.11)
26.3	(27.9)	200	1.24	(0.11)
72.5	(70.3)	150	1.28	(0.07)
Average[a] Stigmastanol Conc. (µg/L)	(SD)	Replicate B N	Diameter	(SD)
Control	(0)	200	1.18t[e]	(0.07)
Blank	(0)	150	1.20t	(0.07)
1.3	(1.4)	100	1.18t	(0.05)
2.5	(2.5)	150	1.17t	(0.11)
8.6	(9.2)	200	1.23	(0.09)
26.3	(27.9)	100	1.17t	(0.09)
72.5	(70.3)	150	1.18t	(0.10)
Average[a] Stigmastanol Conc. (µg/L)	(SD)	Replicate A + B N	Diameter	(SD)
Control	(0)	300	1.19	(0.07)
Blank	(0)	300	1.21	(0.07)
1.3	(1.4)	300	1.21	(0.07)
2.5	(2.5)	300	1.19	(0.09)
8.6	(9.2)	300	1.23*[f]	(0.10)
26.3	(27.9)	300	1.21*	(0.11)
72.5	(70.3)	300	1.23*	(0.10)
		IC25 = >72.6(±70.3) µg/L		

[a] average of 14-17 samples
[b] standard deviation
[c] number of eggs measured
[d] average diameter of eggs in millimeters
[e] significant difference between replicates T-Test ($p \leq 0.05$)
[f] significant difference from the control ($p \leq 0.05$)

3.6.2 *Fathead minnow GSIs*

No significant differences were detected for either male or female fathead minnow GSIs in any of the stigmastanol concentrations during the life-cycle bioassay (Table 15). The IC25s for the male and female fathead minnow GSIs were both >72.6(±70.3) µg/L of stigmastanol (highest concentration tested).

Table 15. Gonad Somatic Indices for Male and Female Fathead Minnows from a Life-Cycle Test with Stigmastanol

		Replicate A					
Average[a]		Male			Female		
Conc. (µg/L)	(SD)[b]	N[c]	GSI[d]	(SD)	N	GSI	(SD)
Control	(0)	4	1.3	(0.1)	6	15.0	(3.3)
Blank	(0)	3	1.5	(0.2)	6	13.7	(2.8)
1.3	(1.4)	4	1.4	(0.4)	8	13.6	(3.0)
2.5	(2.5)	4	1.6	(0.5)	8	14.8	(3.9)
8.6	(9.2)	3	1.4	(0.7)	6	11.7	(3.6)
26.3	(27.9)	4	1.2	(0.3)	7	13.4	(3.3)
72.5	(70.3)	4	1.5	(0.3)	8	14.3	(4.1)
		Replicate B					
Average[a]		Male			Female		
Conc. (µg/L)	(SD)	N	GSI	(SD)	N	GSI	(SD)
Control	(0)	4	1.4	(0.6)	8	12.7	(2.4)
Blank	(0)	4	1.2	(0.3)	6	13.3	(1.7)
1.3	(1.4)	4	1.8	(0.3)	8	14.3	(2.7)
2.5	(2.5)	3	2.1	(0.3)	4	8.8t[e]	(3.6)
8.6	(9.2)	4	1.1	(0.2)	8	13.4	(4.6)
26.3	(27.9)	3	1.5	(0.4)	3	14.8	(3.2)
72.5	(70.3)	4	1.6	(0.3)	8	11.8	(2.1)
		Replicate A + B					
Average[a]		Male			Female		
Conc. (µg/L)	(SD)	N	GSI	(SD)	N	GSI	(SD)
Control	(0)	8	1.3	(0.4)	14	13.7	(2.9)
Blank	(0)	7	1.3	(0.3)	12	13.5	(2.2)
1.3	(1.4)	8	1.6	(0.4)	16	14.0	(2.8)
2.5	(2.5)	7	1.8	(0.4)	12	12.8	(4.7)
8.6	(9.2)	7	1.2	(0.5)	14	12.7	(4.1)
26.3	(27.9)	7	1.3	(0.3)	10	13.8	(3.2)
72.5	(70.3)	8	1.5	(0.3)	16	13.1	(3.4)
		IC25 = >72.6(±70.3) µg/L			IC25 = >72.6(±70.3) µg/L		

[a] average of 14-17 samples
[b] standard deviation
[c] number of samples
[d] average GSI = (Gonad weight ÷ body weight)100
[e] significant difference between replicates T-Test (p≤0.05)

3.6.3 *LSIs*

LSIs of male fathead minnows from all stigmastanol concentrations were not significantly different ($p \leq 0.05$) from LSIs of control fish during life-cycle testing (Table 16). The IC25 for male LSIs was >72.6(±70.3) µg/L stigmastanol.

Table 16. Liver Somatic Indices of Male Fathead Minnows During a Life-Cycle Test with Stigmastanol

Average[a] Concentration (µg/L)	(SD)[b]	N[c]	Replicate A LSI[d]	(SD)
Control	(0)	4	2.4	(0.7)
Blank	(0)	3	2.0	(0.4)
1.3	(1.4)	4	2.5	(0.5)
2.5	(2.5)	4	2.2	(0.5)
8.6	(9.2)	3	2.9	(0.6)
26.3	(27.9)	4	2.5	(0.7)
72.5	(70.3)	4	2.0	(0.4)
Average[a] Concentration (µg/L)	(SD)	N	Replicate B LSI	(SD)
Control	(0)	4	2.0	(0.3)
Blank	(0)	4	2.5	(0.9)
1.3	(1.4)	4	2.1	(0.2)
2.5	(2.5)	3	3.0	(0.7)
8.6	(9.2)	4	2.3	(0.3)
26.3	(27.9)	3	2.8	(0.4)
72.5	(70.3)	4	2.2	(0.5)
Average[a] Concentration (µg/L)	(SD)	N	Replicate A + B LSI	(SD)
Control	(0)	8	2.2	(0.5)
Blank	(0)	7	2.3	(0.7)
1.3	(1.4)	8	2.3	(0.4)
2.5	(2.5)	7	2.5	(0.7)
8.6	(9.2)	7	2.6	(0.5)
26.3	(27.9)	7	2.6	(0.6)
72.5	(70.3)	8	2.1	(0.5)
		IC25 = >72.6(±70.3) µg/L		

[a] average of 14-17 samples
[b] standard deviation
[c] number of samples
[d] (Liver weight ÷ body weight)100

3.6.4 *Condition Factors*

Condition Factors (K) of male and female fathead minnows from all stigmastanol concentrations during the life-cycle bioassay were not significantly different from the Ks of the same sex from the controls, and the IC25s for both male and female Ks were >72.6(±70.3) µg/L stigmastanol (Table 17). There was no dose response relationship detected between stigmastanol concentration and K values for both male and female fathead minnows during the life-cycle bioassay.

Table 17. Condition Factors of Male and Female Fathead Minnows from a Life-Cycle Test with Stigmastanol

		Replicate A					
Average[a]		Male			Female		
Conc. (µg/L)	(SD)[b]	N[c]	K[d]	(SD)	N	K	(SD)
Control	(0)	4	2.0	(0.3)	6	1.6	(0.1)
Blank	(0)	3	1.8	(0.1)	6	1.4	(0.1)
1.3	(1.4)	4	1.7	(0.2)	8	1.6	(0.3)
2.5	(2.5)	4	1.8	(0.2)	8	1.6	(0.2)
8.6	(9.2)	3	2.5	(0.3)	6	1.6	(0.2)
26.3	(27.9)	4	1.8	(0.1)	7	1.5	(0.1)
72.5	(70.3)	4	1.8	(0.2)	8	1.5	(0.1)
		Replicate B					
Average[a]		Male			Female		
Conc. (µg/L)	(SD)	N	K	(SD)	N	K	(SD)
Control	(0)	4	1.7	(0.2)	8	1.5	(0.2)
Blank	(0)	4	1.7	(0.1)	6	1.6t	(0.2)
1.3	(1.4)	4	1.7	(0.1)	8	1.7	(0.1)
2.5	(2.5)	3	1.8	(0.2)	4	1.7	(0.2)
8.6	(9.2)	4	1.3t[e]	(0.2)	8	1.5	(0.1)
26.3	(27.9)	3	1.9	(0.2)	3	1.5	(0.0)
72.5	(70.3)	4	1.8	(0.2)	8	1.5	(0.1)
		Replicate A + B					
Average[a]		Male			Female		
Conc. (µg/L)	(SD)	N	K	(SD)	N	K	(SD)
Control	(0)	8	1.8	(0.3)	14	1.5	(0.2)
Blank	(0)	7	1.8	(0.1)	12	1.5	(0.2)
1.3	(1.4)	8	1.7	(0.1)	16	1.6	(0.2)
2.5	(2.5)	7	1.8	(0.2)	12	1.6	(0.2)
8.6	(9.2)	7	1.9	(0.7)	14	1.5	(0.2)
26.3	(27.9)	7	1.8	(0.1)	10	1.5	(0.1)
72.5	(70.3)	8	1.8	(0.2)	16	1.5	(0.1)
		IC25 = >72.6(±70.3) µg/L			IC25 = >72.6(±70.3) µg/L		

[a] average of 14-17 samples
[b] standard deviation
[c] number of samples
[d] condition factor = 100(total weight as mg ÷ total length as mm)^3
[e] significant difference between replicates (t-test)

4.0 DISCUSSION

Investigators have suggested that phytosterols or their breakdown products may have effects on fish secondary sexual characteristics (Howell et al. 1980; Denton et al. 1985; Howell and Denton 1989; Rosa-Molinar and Williams 1984) or other potential indicators of reproduction effects (MacLatchy and Van Der Kraak 1995). β-sitosterol is usually the most abundant phytosterol measured in pulp mill effluents and has been the focus of many of these studies. However, comparisons between the IC25s for egg production from six fathead minnow life-cycle tests (NCASI 1996, 1997a, 1998, in preparation a, b, c) and compounds measured in the effluents found the highest correlation was with the natural log of the stigmastanol concentration (r^2=0.76, p≤0.03) (NCASI unpublished). Other phytosterols including β-sitosterol (r^2=0.63, not significant at 0.05), campesterol (r^2=0.50, not significant), stigmasterol (r^2=0.28, not significant) and total phytosterols (r^2=0.67, p≤0.05) were not as highly correlated, or were not significantly correlated to the fish reproduction end-point. In addition, Howell and Denton (1989) speculated that stigmastanol and its degradation products caused masculinization of mosquitofish. Because of the high correlation in our earlier life-cycle studies and also because fathead minnow reproduction studies were being performed with β-sitosterol separately (Knutsen et al. 1997), stigmastanol was studied in this fathead minnow life-cycle test and in 7-d fathead minnow and *C. dubia* bioassays.

The amount of stigmastanol detected from samples collected during life-cycle testing was reduced by over 60% from the initial concentration added to the mixing tank (200 µg/L). This reduction was probably caused by stigmastanol adhering to testing apparatus or because of metabolism by bacterial growth on the walls of the test equipment between cleanings. The concentration measured in the tanks averaged 72.5 µg/L in the highest exposure over the entire life-cycle test, which was more than an order of magnitude greater than the highest average stigmastanol concentration calculated to be present at the IC25 during the six life-cycle tests with effluents from pulp or paper mills. The highest stigmastanol concentration in the life-cycle test reported herein fell to approximately 44 µg/L during the two weeks before spawning and the first two weeks of spawning and to about 19 µg/L for the final 4 weeks. Even so, the highest concentrations measured during this life-cycle test immediately before and within the spawning period were four to 10-times greater than the average of the stigmastanol concentrations calculated to have been in the IC25s during the six previous life-cycle tests with effluents. The fish exposed during the life-cycle test reported herein did not show any of the effects on reproduction found with the mill effluents. The number of spawns was similar among all groups, egg production was not affected, and growth of fathead minnows in 7-d or 28-d tests was unchanged when compared to the control or the control blank with the carrier. Thus, stigmastanol did not affect fish survival, growth, reproduction or other bioindicators at concentrations that were four to 26-times greater than concentrations in the IC25 for egg production during earlier life-cycle tests with pulp mill effluents.

These results with stigmastanol were similar to those found by Knutson et al. (1997) for β-sitosterol at concentrations of approximately 60 µg/L. Thus, although these phytosterols are found in pulp mill effluents that affect reproduction at 18% v/v of effluent or greater (NCASI 1996, 1997a, 1998, in preparation a, b, c), stigmastanol and β-sitosterol did not affect fathead minnow reproduction at concentrations that were similar to or much greater than the amounts measured in effluent concentrations that altered fish reproduction. Although the availability of the stigmastanol to the fathead minnows in these tests, compared to its availability in the effluent is unknown, the well water used for dilution had nearly no TOC or TSS, and much less TOC and TSS than the mill effluents. Thus, the percentage of stigmastanol in the dissolved form was probably greater during the tests reported herein than during tests with mill effluents. Since the dissolved form should have greater

bioavailability, the percentage of stigmastanol available to the fish should be greater during this test than in tests with pulp mill effluents (Kukkonen et al. 1991; Gobas et al. 1994). Based on Knutson et al. (1997), and the studies reported here, β-sitosterol and stigmastanol were probably not causative factors in the reduction of egg production observed during the life-cycle tests with mill effluents that were previously completed (NCASI 1996, 1997a, 1998, in preparation a, b, c).

The relatively high correlations of stigmastanol and total phytosterols to reproduction endpoints may indicate a surrogate relationship to other effluent components rather than a cause and effect relationship. NCASI (1998) discussed in more detail the possible sources of effluent components that may cause effects during life-cycle tests, and also reported that polyphenol concentrations, which are a measure of tannin/lignin in the effluent, also correlated highly (r^2=0.76, p≤0.03) with IC25s for egg production. All of these effluent components are originally derived from wood or the pulping process, although they may reach the final effluent through several routes (NCASI, 1998). Thus, because stigmastanol and total phytosterols correlate to IC25 for egg production during fathead minnow life-cycle tests, they may be surrogates for effluent components from pulping that are biologically active. Generally these tests indicate stigmastanol had no effect on fathead minnow survival, growth, or reproduction, or on *Ceriodaphnia* survival and reproduction at concentrations of 16 to 100 µg/L, the highest concentration tested.

5.0 SUMMARY AND CONCLUSION

Fathead minnows (*Pimephales promelas*) and *Ceriodaphnia dubia* were exposed to stigmastanol, a naturally occurring plant phytosterol, during 7-d and life-cycle bioassays. The stigmastanol standard used for all tests was found to be 98.2% pure by GC/MS. A GC^2 acetone carrier was used to dissolve the stigmastanol. The stigmastanol solution was metered into a mixing tank at the rate of 200 µg/L; however, because of instability in solution and the rapid bacterial growth on the testing equipment, average stigmastanol concentrations measured in exposure tanks during life-cycle bioassays were reduced by over 60% from the initial concentrations. Final test concentrations during life-cycle bioassays, although reduced, were above the range of stigmastanol found in most US pulp and paper mill final effluents (NCASI 1997b).

5.1 Bioassay Responses

a) Survival and reproduction of *C. dubia* in all stigmastanol concentrations were not significantly different from the controls during one 7-d bioassay and the IC25s were >99 µg/L of stigmastanol (Table 18).

b) Survival and growth of fathead minnows during one 7-d bioassay were significantly reduced from the control in the control blank with acetone carrier and the highest stigmastanol concentration tested (99.2 µg/L); therefore, the responses were probably due to the carrier and not the stigmastanol (Table 18).

c) The hatchability of eggs spawned in well water and exposed to stigmastanol during the start of a fathead minnow 28-d survival and growth test was significantly reduced at the 13.3(±6.1) µg/L stigmastanol concentration. The percentage of eggs hatching at all higher concentrations of stigmastanol was not altered from the controls and the IC25 was >98.4(±64.5) µg/L stigmastanol (Table 18). Fathead minnow 28-d survival was not significantly different from control survival and the IC25 was >98.4(±64.5) µg/L of stigmastanol. The average weight (growth) of the juveniles at 28-days was significantly greater than the average control weight in the 31.6(±24.7) µg/L concentration of

stigmastanol, but was not changed in any of the other concentrations. The IC25 was >98.4(±64.5) µg/L of stigmastanol (Table 18). The lack of a consistent effect on egg hatchability and growth by stigmastanol, and the finding of no effects at the highest concentrations indicates the responses were probably not related to stigmastanol exposure.

d) Survival and growth during one 56-d fathead minnow bioassay with eggs spawned in well water (a continuation from the first 28-d test) were not significantly different from the controls. IC25s for both survival and growth were >110.9(±83.2) µg/L of stigmastanol.

e) One fathead minnow 28-d growth and survival test using eggs spawned by exposed fish during life-cycle testing resulted in egg hatchability that was significantly reduced from the control in the acetone carrier, the 0.6(±0.3) µg/L, and in the 5.6(±4.2) µg/L test concentrations. Hatchability of eggs at the highest stigmastanol concentration tested of 16(±12) µg/L was not altered from the control, therefore the responses observed at the lower concentrations were probably not stigmastanol related. The IC25 for egg hatchability from exposed life-cycle fish was >16(±12) µg/L of stigmastanol (Table 18). Survival of juvenile fish at 28-days was significantly reduced from the control in the acetone carrier and in the 0.6(±0.3) µg/L stigmastanol concentration and the IC25 was >16(±12) µg/L of stigmastanol (highest concentration tested). Average weights of the larval fish during the test were significantly greater than the control at the same concentrations that reduced survival, and the IC25 was >16(±12) µg/L stigmastanol (Table 18).

f) All egg production parameters from the fathead minnow life-cycle test with stigmastanol including: 1. total eggs produced, 2. total spawns and 3. e/f/d were not significantly different from controls and IC25s were >72.6(±70.3) µg/L stigmastanol (Table 18). Adult survival of male and female fathead minnows to the end of the life-cycle test were not altered from the control survival and the IC25s were >72.6(±70.3) µg/L stigmastanol. There were significant differences between survival of male and female fish within a concentration. This was probably caused by aggressiveness of the males, resulting in mortality of the female fish. Average final weights of male and female fish from all concentrations of stigmastanol at the end of the spawning period were not significantly different compared to control fish ($p \leq 0.05$), and the IC25s were >72.6(±70.3) µg/L stigmastanol (Table 18). Hatchability of fathead minnow eggs produced from fish exposed to the same stigmastanol concentration was significantly different from control fish in the 2.5(±2.5) µg/L stigmastanol concentration. However, egg hatchability at all higher concentrations of stigmastanol was not altered. The lack of a consistent effect on hatchability by stigmastanol and no effect on hatching of eggs from the higher concentrations indicates stigmastanol concentrations up to 72.6 µg/L had no effect on egg hatchability.

5.2 Biomarker Results

a) Egg diameters were very uniform and differences of only 2% between groups were detected as significant. Eggs collected during life-cycle testing from fish in 8.8(±9.2) µg/L, 26.2(±27.9) µg/L, and 72.6(±70.3) µg/L stigmastanol were significantly larger than control eggs. The IC25 for egg diameter was >72.6(±70.3) µg/L of stigmastanol (Table 18). Egg diameters were significantly different in 6 of 7 comparisons between the two replicates including the acetone carrier. The very small differences detected as significant and the frequent significant differences between replicates indicates the differences observed in egg size were probably not due to the stigmastanol, and are unlikely to have population level effects.

b) No significant differences were detected for either male or female fathead minnow average GSIs in any of the stigmastanol concentrations during the life-cycle bioassay. The IC25 for the male and female fathead minnow GSIs were all >72.6(±70.3) µg/L of stigmastanol.

c) LSIs of male fathead minnows from all stigmastanol concentrations were not significantly different from LSIs of control fish ($p \leq 0.05$) during life-cycle testing. The IC25 for male LSI was >72.6(±70.3) µg/L of stigmastanol.

d) Condition Factors (K) of male and female fathead minnows from all stigmastanol concentrations during the life-cycle bioassay were not significantly different from the Ks of the same sex from the controls, and the IC25s for both male and female Ks were >72.6(±70.3) µg/L of stigmastanol.

5.3 Conclusion

Stigmastanol had no effects on any measured endpoint at concentrations ranging from 3- to over 20-times greater than the average concentration of 4.8 µg/L calculated to be in the IC25 concentrations of effluent from the six previous life-cycle tests. Thus, stigmastanol is probably not a causative factor in the results of laboratory studies of effects of the pulp and paper mill effluent on fish reproduction; however, the high correlation observed previously indicates this compound may be a surrogate for other biologically active compounds originating in the pulping process.

Table 18. Summary of Results from Bioassays with Stigmastanol

Test and Endpoint	IC25 µg/L Stigmastanol
Ceriodaphnia dubia 7-d bioassay	
Survival	>99.2
Reproduction	>99.2
Fathead minnow 7-d bioassay	
Survival	97.2[a]
Growth	95.8[a]
Fathead minnow 28-d bioassay (unexposed eggs)	
Hatchability	>98.4(±64.5)
Juvenile survival	>98.4(±64.5)
Juvenile growth	>98.4(±64.5)
Fathead minnow 28-d bioassay (eggs from exposed fish)	
Hatchability	>16(±12)[b]
Juvenile survival	>16(±12)[c]
Juvenile growth	>16(±12)
Fathead minnow 56-d bioassay (continued from first 28-d)	
Juvenile survival	>110.9(±83.2)
Juvenile growth	>110.9(±83.2)
Fathead minnow life-cycle bioassay	
Average total eggs	>72.6(±70.3)
Total spawns	>72.6(±70.3)
Eggs/F/Day	>72.6(±70.3)
Egg hatchability	>72.6(±70.3)[d]
Adult survival	>72.6(±70.3)
Final weight	>72.6(±70.3)
Biomarkers	
Egg size	>72.6(±70.3)
Gonad somatic index-males	>72.6(±70.3)
Gonad somatic index-females	>72.6(±70.3)
Liver somatic index-males	>72.6(±70.3)
Condition factors-males	>72.6(±70.3)
Condition factors-females	>72.6(±70.3)

[a] acetone carrier and highest stigmastanol concentration significantly reduced from control, effect not due to stigmastanol

[b] acetone carrier, 0.6(±0.4), and 5.7(±4.3) µg/L stigmastanol significantly reduced from control

[c] acetone carrier and 0.6(±0.4) µg/L stigmastanol were significantly reduced from control

[d] significantly reduced from the control at 2.5(±2.5) µg/L stigmastanol only

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APPENDIX A

PHYTOSTEROL CONCENTRATIONS OF SAMPLES TAKEN FROM THE HIGHEST EXPOSURE CONCENTRATION DURING LIFE-CYCLE BIOASSAYS WITH STIGMASTANOL

Table A1. Phytosterol Concentrations of Samples Taken During Life-Cycle Bioassays with Stigmastanol, 08/18/97-12/23/97

Date	Campesterol µg/L	Stigmasterol µg/L	β-Sitosterol µg/L	Stigmastanol µg/L
Units				
08/20/97	ND[a]	ND	1.8	106.1
08/26/97	ND	ND	0.9	56.3
09/03/97	ND	ND	1.8	95.2
09/09/97	ND	ND	2.3	190.6
09/17/97	2.2	ND	1.6	51.4
09/23/97	ND	ND	3.7	273.3
09/30/97	ND	ND	1.3	65.6
10/07/97	ND	ND	1.8	125.5
10/16/97	ND	ND	ND	29.4
10/28/97	ND	ND	0.6	42.4
11/05/97	ND	ND	0.8	23.9
11/12/97	2.0	ND	1.4	72.7
11/18/97	ND	ND	2.4	36.1
11/25/97	ND	ND	ND	9.6
12/02/97	ND	ND	1.5	15.2
12/09/97	NA[b]	NA	NA	33.7[c]
12/16/97	ND	ND	ND	6.5

[a] not detected
[b] not available
[c] calculation based on 35% of headbox concentration

APPENDIX B

AVERAGE PHYTOSTEROL CONCENTRATIONS OF SAMPLES TAKEN FROM THE HIGHEST EXPOSURE CONCENTRATION DURING EACH TEST PERIOD

Table B1. Phytosterol Concentrations From One Sample of Stigmastanol Taken During a Fathead Minnow (*Pimephales promelas*) and (*Ceriodaphnia dubia*) 7-Day Test Period From 03/19/98 to 03/25/98

Phytosterol	N[a]	Units	Concentration
Campesterol	1	µg/L	4.2
Stigmasterol	1	µg/L	ND[b]
β-Sitosterol	1	µg/L	1.7
Stigmastanol	1	µg/L	99.2

[a] number of samples
[b] not found above detection limit

Table B2. Average Phytosterol Concentrations of Samples Taken from the Highest Exposure Concentration During Each Fathead Minnow Early Life-Stage Exposure with Stigmastanol Using Eggs Obtained From Well-Water Cultures

	28-d Test 1 08/21/97 to 09/18/97			56-d Test 2 08/21/97 to 10/16/97		
Phytosterol	N[a]	AVG[b]	SD[c]	N	AVG	SD
Campesterol	4	0.6	1.1	8	0.3	0.8
Stigmasterol	4	ND[d]	NA[e]	8	ND	NA
β-Sitosterol	4	1.7	0.6	8	1.7	1.1
Stigmastanol	4	98.4	64.5	8	110.9	83.2

[a] number of samples
[b] average phytosterol in µg/L
[c] standard deviation
[d] not found above detection limit
[e] not applicable

Table B3. Average Phytosterol Concentrations of Samples Taken from the Highest Exposure Concentration During a Fathead Minnow Early Life-Stage Bioassay Using Eggs Obtained from Exposed First Generation Fish

	28-d Bioassay 11/25/97 to 12/23/97		
Compound	N[a]	AVG[b]	(SD)[c]
Campesterol	4	ND[d]	NA[e]
Stigmasterol	4	ND	NA[e]
β-Sitosterol	4	0.5	0.9
Stigmastanol	4	16	12.2

[a] number of samples
[b] average phytosterol in µg/L
[c] standard deviation
[d] not detected above detection limit
[e] not applicable

APPENDIX C

FATHEAD MINNOW REPRODUCTION RESULTS DURING LIFE-CYCLE TESTING WITH STIGMASTANOL

Table C1. Fathead Minnow Reproduction Results During Life-Cycle Testing with Stigmastanol Using Eggs Spawned in Well Water from 08/18/97 to 12/23/97

Stig. Conc. µg/L			Average	DAYS		TOTAL	EGGS/	EGGS/	EGGS/F	MEAN. EGGS	MEAN EGGS		MEAN
Avg.[a]	(SD)[b]	REPL.	Female	2F	1F	Eggs	Spawn	Spawn	Day	Per Conc.	Per Spawn	E/F/D	% Hatch
0.0	(0)	ControlA1	2	42	0	2781	15	185.40	33.11				
0.0	(0)	ControlA2	2	42	0	2292	17	134.82	27.29				
0.0	(0)	ControlA3	2	42	0	3162	11	287.45	37.64	2802	204.35	34.06	71.8
0.0	(0)	ControlA4	1.63	22	13	994	11	90.36	17.42				
0.0	(0)	ControlB1	2	42	0	3481	18	193.39	41.44				
0.0	(0)	ControlB2	2	42	0	3316	10	331.60	39.48				
0.0	(0)	ControlB3	2	42	0	3431	16	214.44	40.85				
0.0	(0)	ControlB4	2	42	0	2960	15	197.33	35.24				
0.0	(0)	BlankA1	OUT										
0.0	(0)	BlankA2	2	42	0	2378	14	169.86	28.31				
0.0	(0)	BlankA3	2	42	0	2903	12	241.92	34.56	2145	181.97	26.18	61.8
0.0	(0)	BlankA4	2	42	0	2742	16	171.38	32.64				
0.0	(0)	BlankB1	1.83	35	7	2776	14	198.29	36.12				
0.0	(0)	BlankB2	2	42	0	1661	9	184.56	19.77				
0.0	(0)	BlankB3	2	42	0	2030	10	203.00	24.17				
0.0	(0)	BlankB4	1.62	26	16	524	5	104.80	7.70				
1.3	(1.4)	A1	2	42	0	3146	16	196.63	37.45				
1.3	(1.4)	A2	2	42	0	2620	7	374.29	31.19				
1.3	(1.4)	A3	2	42	0	2805	17	165.00	33.39	2788	207.99	33.19	70.8
1.3	(1.4)	A4	2	42	0	3384	15	225.60	40.29				
1.3	(1.4)	B1	2	42	0	3285	17	193.24	39.11				
1.3	(1.4)	B2	2	42	0	1710	10	171.00	20.36				
1.3	(1.4)	B3	2	42	0	1720	11	156.36	20.48				
1.3	(1.4)	B4	2	42	0	3636	20	181.80	43.29				
2.5	(2.5)	A1	2	42	0	2939	18	163.28	34.99				
2.5	(2.5)	A2	2	42	0	2988	14	213.43	35.57				
2.5	(2.5)	A3	2	42	0	1501	7	214.43	17.87	2497	191.02	29.72	58.8
2.5	(2.5)	A4	2	42	0	3473	16	217.06	41.35				
2.5	(2.5)	B1	2	42	0	1305	8	163.13	15.54				
2.5	(2.5)	B2	2	42	0	2255	15	150.33	26.85				
2.5	(2.5)	B3	OUT										
2.5	(2.5)	B4	2	42	0	3017	14	215.50	35.92				
8.8	(9.3)	A1	2	42	0	1023	6	170.50	12.18				
8.8	(9.3)	A2	OUT										
8.8	(9.3)	A3	2	42	0	1729	12	144.08	20.58	1912	162.35	22.76	63.3
8.8	(9.3)	A4	2	42	0	702	7	100.29	8.36				
8.8	(9.3)	B1	2	42	0	703	7	100.43	8.37				
8.8	(9.3)	B2	2	42	0	2710	11	246.36	32.26				
8.8	(9.3)	B3	2	42	0	3298	19	173.58	39.26				
8.8	(9.3)	B4	2	42	0	3219	16	201.19	38.32				
26.2	(27.9)	A1	2	42	0	2150	15	143.33	25.60				
26.2	(27.9)	A2	1.98	41	1	1520	13	116.92	18.28				
26.2	(27.9)	A3	2	42	0	2975	18	165.28	35.42	2165	169.72	27.84	62.8
26.2	(27.9)	A4	2	42	0	3541	17	208.29	42.15				
26.2	(27.9)	B1	OUT										
26.2	(27.9)	B2	2	42	0	206	3	68.67	2.45				
26.2	(27.9)	B3	2	42	0	3506	15	233.73	41.74				
26.2	(27.9)	B4	1.72	18	7	1259	5	251.80	29.28				
72.5	(70.3)	A1	2	42	0	2692	13	207.08	32.05				
72.5	(70.3)	A2	2	42	0	2636	16	164.75	31.38				
72.5	(70.3)	A3	2	42	0	1109	9	123.22	13.20	2492	175.32	29.66	60.6
72.5	(70.3)	A4	2	42	0	1147	10	114.70	13.65				
72.5	(70.3)	B1	2	42	0	2703	12	225.25	32.18				
72.5	(70.3)	B2	2	42	0	3274	16	204.63	38.98				
72.5	(70.3)	B3	2	42	0	3642	18	202.33	43.36				
72.5	(70.3)	B4	2	42	0	2730	17	160.59	32.5				

[a] average of 14-17 samples
[b] standard deviation