EFFECTIVE SPAWNING STRATEGIES FOR PRODUCING VIABLE FATHEAD MINNOW EMBRYOS FOR USE IN FISH EMBRYO TOXICITY TESTS

By

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ABSTRACT

Traditionally, the toxicity of chemicals and industrial effluents is evaluated using a 7-day fathead minnow (*Pimephales promelas*) larval growth and survival (LGS) test. However, there has been a push to adopt an alternative toxicity testing method, the fish embryo toxicity (FET) test, which utilizes fathead minnow embryos rather than larvae. The adoption of the FET test as a replacement for the LGS test is considered to represent an improvement in animal welfare as embryos are thought to be less sensitive to pain and distress than older organisms. Implementation of the FET testing protocol has encountered some challenges, namely, the difficulties associated with obtaining the number of embryos required to initiate the test. To aid in the adoption of the FET test, this study sought to optimize the breeding output of fathead minnows through manipulation of male: female ratios and breeding structure availability. To identify which sex ratio produced the largest number of embryos, breeding output was monitored in minnow breeding colonies with minnows arranged in 1:2, 1:4, 2:4 or 2:8 male: female ratios with limited (2 hr.) or unlimited (24 hr.) access to breeding structures. Over the course of 28 days, when access to breeding structures was limited, the 1m: 2 ratio produced a mean of 9.9 embryos/colony/day. The 1m: 4f ratio produced a mean of 22 embryos/colony/day. The 2m: 4f ratio produced a mean of 1.8 embryos/colony/day. And, the 2m: 4f ratio produced 3.7 embryos/colony/day. This trend was similar when access to breeding structures was unlimited with embryo counts respectively at 33.9, 58.3, 35.4, and 42.2 embryos/colony/day. The results of this study demonstrated that spawning was maximized through use of a 1:4 male: female ratio. Additionally, it was determined that more uniform embryo development was observed with limited access to breeding

structures. By using a 1 male: 4 female sex ratio and providing limited access to breeding structures, investigators will be able to optimize fathead minnow embryo production, thus making the process of FET initiation more efficient.

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Toxicity testing is mandated by governmental regulatory agencies in the United States and European Union to determine how substances affect organisms [1,2]. Per these legislative demands, industry is required to test their effluent's toxicity by evaluating potential lethal and sub-lethal consequences of their effluents to animals. Currently, toxicity testing methods in the United States use fish as model organisms, with the fathead minnow (*Pimephales Promelas*) being among those most commonly used [3,4]. The present protocol for the larval growth and survival toxicity test (LGS) involves dosing newly hatched larvae at various chemical concentrations and recording mortality and resulting growth over the course of 7 days [5]. Despite the effectiveness of the LGS at evaluating industrial effluents, there are growing public concerns and legislative mandates aimed at creating a more ethical test with regards to animal welfare [6].

To make a more ethical test that addresses these animal welfare concerns, researchers have developed the fish embryo toxicity test (FET) as a potential alternative to the LGS. The FET test utilizes fathead minnow embryos as the test subject rather than larvae. The use of embryos is thought to improve animal welfare as embryos are considered less sensitive to pain and distress than their larval counterparts [7-9].

While this test seems to meet the new criteria for toxicity testing, there are functional issues that need to be addressed before its adoption. The FET test protocol calls for 168 embryos at a developmental stage of 32 cells or less [10]. This developmental requirement is stringent because after the 32-cell stage, the chorion of the embryo becomes water-hardened [11]. This prevents or severely limits the ability of test chemicals and effluents from penetrating the embryo, greatly obscuring FET results [12].

Embryos less than 32 cells are defined as “viable” for FET test initiation. It has been determined that fathead minnow embryos require 2.83 hours to reach the 32-cell stage [11]. This is problematic because this is a narrow timetable for investigators to collect embryos, observe their developmental stage, sort the viable embryos from the nonviable, and dose the viable embryos. These strict mandates for test initiation put a strain on researchers ability to begin a test [12,13]. Frequently, investigators gather embryos but are unable to progress through the steps of test initiation before the embryos are no longer viable.

In order for the FET test to be effectively implemented as a viable alternative to the LGS test, the process of initiating a successful test must become more time efficient and effective [12]. As such, the goal of this study was to optimize embryo production. Specifically, the objectives of this study were 1) to determine what sex ratio (males: females) yields the largest quantity of embryos and 2) evaluate embryo developmental stage when breeding structures are accessible for 2 or 24 hours.

MATERIAL AND METHODS

General Animal Husbandry:

Throughout the 28-day study, photoperiod remained constant (16 hours of light: 8 hour dark), fish were fed flake food twice daily (8:30am and 6:00pm), and tanks were cleaned daily after embryo collection. Cleaning entailed maintaining consistent water temperature (~25.5°C), siphoning off feces and a third of the tank's water volume, and restoring tank volume with same temperature, dechlorinated tap water. Tanks were aerated constantly throughout the day. Additionally, when mortality was observed, dead minnow were replaced with sexually mature minnows stocked at Texas Christian University to maintain ratios.

Experimental Design:

In order to determine the most productive breeding ratios, these ratios' spawning activity needed to be observed for an extended amount of time. Achieving this goal requires that 8 five-gallon tanks be reserved for 1 male: 2 female, 6 ten-gallon tanks be reserved for the 1m: 4f ratio; and 6 twenty-gallon tanks be reserved for the 2m: 4f and 2m: 8f ratio's. This allowed at minimum 1.66 gallons per minnow. Sexually mature fathead minnows were obtained from breeding stocks held at Texas Christian University. Females used had no prior breeding experience. After minnows were moved into tanks and prior to experimentation, no breeding structures, halved 3 inch diameter polyvinyl chloride pipes, were added to the tanks. This prevented the fish from spawning, thus allowing a distinct start to the experiment. During experimentation, number of breeding

structures in a tank was proportional to the number of males in a tank to decrease competition for breeding sites. The experiment was designed to last 28 days.

Embryo Collection and Assessment of Developmental Stage:

On the first day of data collection, breeding structures were added to each of the tanks. After two hours of breeding time, embryos were counted and a random sample of approximately 20 embryos was collected to determine developmental stage. After collection, breeding structures were removed from half of the tanks of each ratio. The other half of the tanks had structure available for 24 hours. After day 14 of embryo collection, the tanks that had breeding structures available for 2 hours were switched to a 24-hour availability. Conversely, the tanks with breeding structures available for 24 hours were switched to a 2-hour availability. Once collected, the random sample of embryos was put on ice to slow development and then transported to a Leica DMi1 microscope to assess developmental stage.

Statistical Analysis:

Statistical analysis was done through one-way ANOVA and Tukey's tests to determine if differences seen between clutch sizes and FET viability were significant due to breeding ratio and structure availability. A 95 percent confidence interval was used to determine significance.

RESULTS

Determination of the Most Productive Breeding Ratio

When breeding structures were available for 2 hours, the 1m: 4f breeding ratio produced the largest number of embryos, 22 embryos per day where the 1m: 2f produced 9.9 embryos per colony per day, the 2m: 4f produced 1.8 embryos, and the 2m: 8f ratio produced 3.7 embryos per day (Fig.1, ANOVA, p=0.01).

When breeding structures available for 24 hours, there is no statistical difference in the number of embryos produced between the different breeding ratios. However, it should be noted that the 1m: 4f breeding ratio still produced the largest number (58.3 embryos per day) (Fig.1, ANOVA, p=0.22).

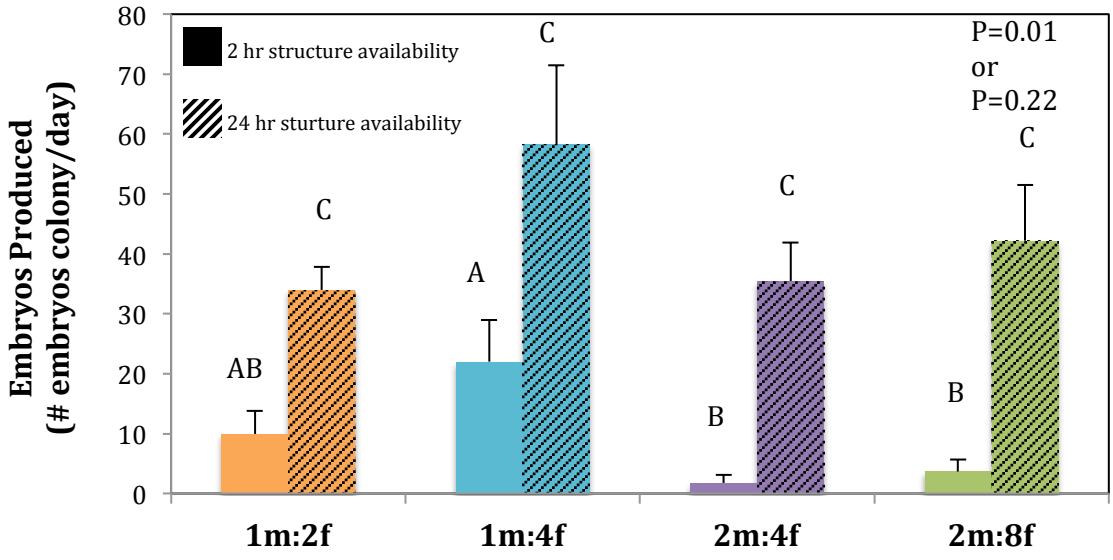

Figure 1. The number of embryos produced when structures were available for 2 and 24 hours under different male (m) to female (f) breeding ratios. Letters represent statistically significant differences. If similar letters are located above the bars, then the values are statistically similar. Different letters above bars represent statistically different values. Error bars represent the standard errors.

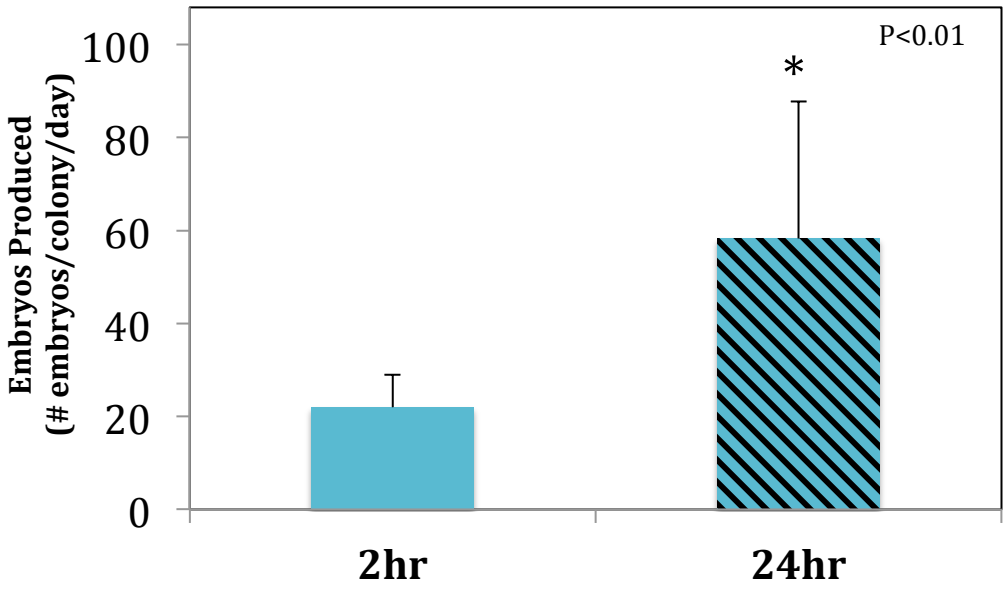

Figure 2. The number of embryos produced by 1 male: 4 female colonies given access to breeding structures for 2 and 24 hours. * represents statistically significant differences. Error bars represent the standard errors.

Determination of Breeding Structure Availability

When investigating the effects of manipulation of breeding structure availability, similar results were seen across all ratios. It was observed that all ratios saw a significant increase in embryo production, as breeding structures were available for a longer amount of time (Fig.1, ANOVA, P=0.01). The data shown for the 1m: 4f ratio is a representative of all ratios. (Fig. 2, ANOVA, P<0.01) (Fig. 3, ANOVA, P<0.01).

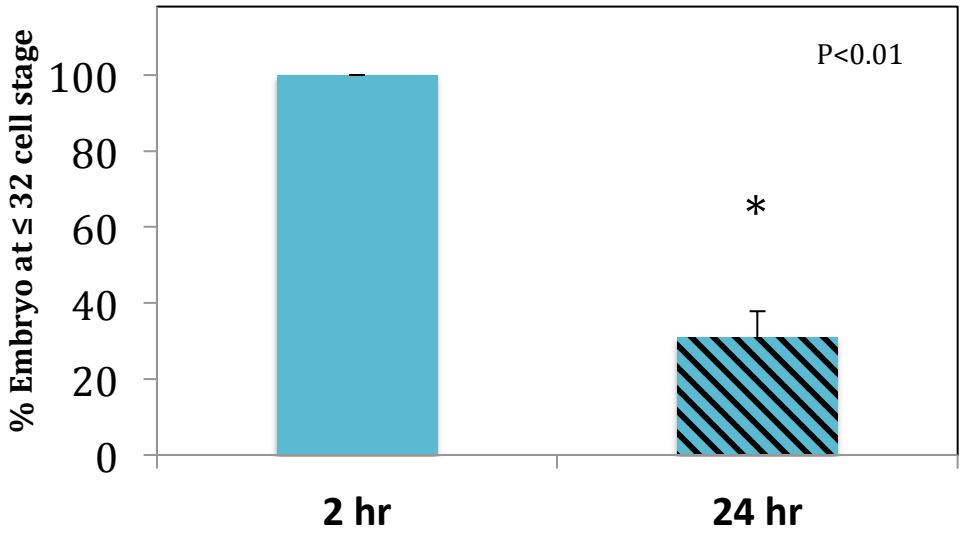

Figure 3. Percent of embryos viable for use in the FET test (less than or equal to 32 cell stage) when collected from minnow colonies with either 2 or 24 hours access to breeding structures. *represent statistically significant differences. Error bars represent the standard errors.

When comparing the two breeding structure availabilities investigated, the embryos that were laid on the 2 hour available structures were all less than or equal to 32 cells. Therefore, 100% of these embryos are viable for FET initiation (Fig 3, ANOVA, $p<0.01$). When looking at the embryos found on the 24 hour available structures, it was observed that 30.9% of the embryos were less than or equal to 32 cells and not viable for FET initiation (Fig 3, ANOVA, $p<0.01$).

DISCUSSION

Male-Male Competition's Role in Breeding

The original hypothesis was that the 2m: 8f ratio would produce the largest number of embryos. This was hypothesized given the fact that a larger number of females allows for increased egg production potential. However, the 2m: 4f did not produce the largest number of embryos. The 2m: 8f and 2m: 4f ratio produced a statistically smaller numbers of embryos over the 28 day study when compared to the 1m: 4f ratio. It was hypothesized that this could be due to increased competition between the males since they are territorial species [3,14]. It was observed during the course of the experiment that the males seemed very aware of each other, frequently making aggressive moves (i.e. bumping each other). Jacob et al (2009) found that *Phoxinus phoxinus*, another minnow species, developed dominance hierarchies that affected spawning activity between males. The more dominant a male then the more reproductive success that male experienced [15]. Do to the similarities between *Phoxinus phoxinus* and fathead minnows, it is conceivable that more time and energy was directed to male/male competition rather than breeding, thus decreasing the colony's breeding output [15, Sellin Jeffries M, TCU, Fort Worth, TX, USA, m.jeffries@tcu.edu].

Fathead minnows are thought to exhibit allopaternal care, meaning the males take care of the embryos. Recent studies has shown that female fathead minnows prefer to mate with males that already have embryos in their nests [16,17]. When applying this data to the data of this study, the 1m: 4f ratio could be the most productive sex ratio because since there is only one male, his is the only nest that will have embryos. If this is

an attractive quality for female fathead minnows, then there is a greater chance that this singular male will continue to breed.

Increased Efficiency for the FET Test

The data shows that for the 1m: 4f sex ratio produced twice as many embryos, and therefore, is the best gender ratio for producing embryos. However, only the embryos gathered during the 2-hour breeding structure availability was statistically significant. Practically, the goal was to optimize embryo production so that FET tests can be initiated. The difference between the 2-hour and 24-hour breeding structure availability, albeit not statistically significant, could provide the necessary breeding output to start a FET test, rather than waste the embryos that fail to complete a 168 embryo test group. Additionally, by using a 1m: 4f ratio, investigators have the ability to split a 10-gallon tank, thus doubling the opportunity to gather embryos per tank.

This data suggests that a 1m: 4f sex ratio maximizes embryo production, but can also become a more efficient means of gathering embryos for FET test initiation by utilizing the 2-hour breeding structure availability. Instead of constantly having tasks interrupted in order to check for embryos, a lab can now know exactly when is the optimal time to gather embryos and being FET tests. This allows for better planning of laboratory time, thus increasing efficiency. Using the 2-hour breeding structure availability aids in the efficiency of FET initiation because all embryos are viable for use in the FET. When using a 24-hour breeding structure availability when gathering embryos for a FET test, significant time is spent individually sorting through the embryos to isolate the ~30% that are viable for FET initiation. This process is extremely

inefficient because during this sorting, the embryos are continuously dividing. Therefore, there is potential for previously viable embryos to develop into nonviable embryos during this sorting time.

Additional Studies

While this experiment gives a definitive preferred breeding strategy amongst the ones tested, further research is needed to evaluate the productivity of more sex ratios. We were only able to evaluate the effectiveness of four different ratios. A previous study conducted by Gordon et al. (2014), which focused on optimization of large scale embryo production, used an 8 male: 60 female sex ratio [18]. Despite the introduction of multiple males, they had success at generating large quantities of embryos. This leads to the question that can having more females per male minimize the effects of the male/male competition that were observed in our study.

Due to the hypothesis of male/male competition decreasing spawning in tanks with multiple males, it should then be evaluated to see if there is a threshold to the breeding potential of a singular male by increasing the number of females in the tank (i.e. 1male: 8 females). Further experimentation could show that more embryos could be produced in tanks with 5+ females in a tank due to no male-male competition and a greater egg laying potential. If so, this would then be the optimal sex ratio to use when breeding fathead minnows for FET test initiation. Additionally, further investigation into whether tanks with two males of comparable dominance scores or tanks of males with different dominance scores would yield different breeding results.

This experiment manipulated sex ratio and breeding structure availability to determine how to gather the most embryos. An additional variable that could be manipulated is the breeding structure. Weldon et al (2012) conducted a study that explored the effects on embryo production of different surface areas of breeding substrates. They found that there were no differences in breeding between the breeding substrates with the largest surface area (225 cm^2) and with the lowest (85cm^2) [19]. However, a different study conducted by Wisenden et al (2009) showed significant differences in embryo deposition when fatheads had either lily pads (high surface area) or submerged sticks (low surface area). The data showed that lily pads contained more clutches and more embryos, hinting to a preference of breeding substrate by the fathead minnow [20]. These two studies yielded different results, so it would be useful to consider this variable for indoor, tank-based breeding.

Application to Other Modes of Research

While this information is specifically important to the FET and its adoption as a valid method of toxicity testing, the fathead minnow is an important model organism in toxicology and, as a result, this data is significant to many investigators. For example, any researcher conducting fathead minnow early life stage development studies will find this useful [3,21,22]. These studies need large numbers of embryos/larvae to answer their research question; therefore, using a 1m: 4f ratio can increase efficiency of beginning these experiments.

CONCLUSION

Based on previous investigation into the validity and implementation of the FET, researchers are calling for an increased efficiency of procuring viable embryos [12,13]. In order for the FET to become adopted, measures to increase the efficiency of test initiation must be taken. By utilizing a 1m: 4f, researchers should have a greater production of embryos, making FET initiation more efficient.

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