Spawning Conditions Affect Clutch Probability and Size in Laboratory-Housed Zebrafish (*Danio rerio*)

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ABSTRACT

Zebrafish are common experimental models used in biological studies that are bred and raised in laboratory settings. Published studies, anecdotal evidence, and industry practices are variable and offer conflicting suggestions on maximizing reproductive success, particularly regarding sex ratios and segregating males and females before spawning. This study identified conditions that promote maximum reproductive success (clutch probability and average clutch size) in zebrafish. Clutch probability was higher when females were seven to ten months old and bred in groups with equal sex ratios and an artificial spawning substrate in the winter or spring. Clutch size was significantly larger when females were seven to ten months old, outnumbered by males, and bred with an artificial spawning substrate. Optional spawning substrates (marbles and plants) improved reproductive success, whereas other parameters had no impact. These data support the implementation of simple steps that reliably maximize reproductive success of laboratory zebrafish.

KEY WORDS Reproduction; Breeding; Seasonality; Behavior; Substrate; Sex Ratios; Captivity; Eggs

INTRODUCTION

Zebrafish (*Danio rerio*) are commonly used experimental models in biological research and biomedicine.[1, 2] Zebrafish are small, tropical freshwater fish that can tolerate a wide variety of environmental conditions and breed in captivity,[1] which contributes to their popularity in the laboratory. Zebrafish mature quickly and can produce thousands of eggs in their lifetime.[1] Reliable generation of large batches of eggs is essential for the success of zebrafish as a model organism. Reproductive success can be measured in several ways, and most often refers to the frequency and size of viable clutches. Several factors affect reproductive success and can be grouped into those related to health and husbandry, social factors, and spawning habitat. Unsurprisingly, parameters that affect fish health[3] including water quality,[4] diet,[5] and parasite load[6] all affect reproductive success. This analysis focused on spawning habitat and social factors that are purported to increase reproductive success in otherwise healthy fish, though the parameters tested may also be applicable in compromised populations.

Social factors

Clutch size is affected by the age and size of fish. Laboratory-raised zebrafish reach sexual maturity in 3–6 months,[1] though fish aged 7–18 months lay more eggs than do younger or older fish,[7] with best reproductive success attained while fish are 6–12 months.[3] Young adults may lay poorer quality eggs[3] than more mature fish. Fish body size is a factor in reproductive success – larger females lay larger and more frequent clutches.[8–10] Female size differences may be strictly due to larger females being able to physically produce greater numbers of eggs, though reproductive success also differs by social rank in which territorial females reproduce more successfully than other females in their territory.[11] As a shoaling species, social factors influence zebrafish reproductive success. Sex ratios and housing density of zebrafish affect male territoriality, which may affect reproductive success.[12] Females are selective and spawn more frequently when paired with a certain male, but their preferences do not correlate with male dominance.[9] Wild zebrafish reproduce in pairs,[13] while laboratory-housed fish are often spawned in groups.[7, 14, 15] Some have reported that group spawning and pair spawning in laboratory populations are equally successful,[16, 17] though most sources suggest setting up spawning groups with females outnumbering males to increase the number of eggs generated.[7, 12, 18]

The suggested practice of some researchers and tank manufacturers is to segregate males and females overnight using clear dividers that are removed when the lights are turned on in the morning[4, 19, 20] or at the desired breeding time. This is intended to serve two purposes. First, dividers separate sexes to allow for controlled timing of breeding. Second, dividers prevent fish from interacting while allowing them to process olfactory and visual displays as a way to increase spawning behaviors, because zebrafish reproductive behavior is influenced by both olfactory and visual cues.[21, 22] Ovarian steroid glucuronides serve as sex attractants for males.[23] Male zebrafish release pheromones that trigger female ovulation and in turn, females release a pheromone that triggers courtship behavior in males.[1] Common practice suggests selecting the most vibrant and colorful males for successful breeding,[22] though some evidence suggests that female choice may not depend on appearance, but rather on male personality.[24] It is unclear if the use of dividers increases reproductive success related to these displays or is simply a useful mechanism to regulate breeding timing.

Spawning habitat

Laboratory spawning is meant to mimic certain factors that are associated with zebrafish spawning in the wild. Reproduction of both wild-strain and laboratory zebrafish occurs exclusively in the morning for roughly two hours beginning at "dawn" (when the system lights turn on).[7, 13] Wild zebrafish spawn seasonally, and laboratory colonies are maintained at temperatures and light cycles that mimic this breeding season to allow for generation of eggs year-round.[7] Indeed, light pollution negatively influences reproductive success.[25] Seasonal breeding may correlate with diet that changes with the seasons, rather than season per se, as wild-caught zebrafish breed year-round in a controlled setting.[15] In the wild, zebrafish tend to inhabit shallow ponds, ditches, and slow-flowing streams,[15] so laboratory spawning is typically set up in shallow tanks. Spawning in too small volumes (≤ 200 mL) negatively affects reproductive success.[14, 26] Wild zebrafish also spawn around plants[13] and after heavy downpour,[15] which is mimicked in the lab by changing the water in the spawning tank at dawn (this also serves to clean the water into which eggs are laid). Factors purported to promote large clutch sizes include using a substrate such as marbles in the spawning tank.[12, 17, 27] Similarly, incorporating live plants into zebrafish housing may have positive impacts on health and stress,[28, 29] and thus reproduction.

Despite such common use, there is insufficient experimental data to support recommended best practices to increase spawning success in laboratory-housed zebrafish. Though many sources outline factors that are purported to aid in reproductive success contradictions abound. These factors include characteristics of the fish (age, health, size), social factors (number and sex ratio of breeding fish, housing density, social dominance, pheromone cues), and environmental factors (light duration, intensity, temperature, season, substrate, habitat). These experiments consist of retrospective analyses bolstered by controlled spawning to probe for and test correlations in reproductive success to identify variables associated with clutch production and size that can be readily implemented in zebrafish colonies.

METHODS AND PROCEDURES

Animal husbandry

Zebrafish husbandry and all experimental procedures were approved by the Murray State Institutional Animal Care and Use Committee, protocol #2017-024. Adult zebrafish (*Danio rerio*) were raised in house from the AB and EKK strains which were originally purchased from the Zebrafish International Resource Center (Eugene, OR) and Ekkwill Waterlife Resources (Ruskin, FL) respectively. Fish were housed in a recirculating rack system (Aquaneering, San Diego, CA) with a 14:10 h light: dark cycle in tanks of mixed sexes at a density of five fish or fewer per L (though juveniles are stocked at higher densities based on age and size). Water quality was continuously monitored using Neptune APEX (Morgan Hill, CA). The pH ranged between 7.5 and 8 and water temperatures were kept at 27.5°C ± 1. The zebrafish were fed twice daily with adult zebrafish diet (Ziegler, East Berlin, PA).

Basic spawning parameters

Ages of spawned fish ranged from 2–25 months old. Individuals were set up to spawn at most twice a week. Fish were set up in 1 L breeding tanks with a slotted liner to segregate eggs from adults (Aquaneering) after the last feeding in the evening (5 pm). Clear plastic dividers were purchased as part of the spawning tank assembly (Aquaneering) and fit into grooves inside the slotted tank liner. A single floating broad-leaf green plastic aquarium plant approximately 7 cm long was used to provide cover to zebrafish. When plants were included together with dividers, females were placed in the plant side of the spawning tank. A single layer of assorted marbles (blue, green, clear, and yellow) per tank was used to mimic rocky substrate. When marbles and dividers were both present, marbles were distributed evenly on each side of the divided tank.

Two locations were used for spawning: the fry incubator and the standard housing room rack system. Both locations were kept on 14:10 h light:dark cycles. The housing room dawn was 9 am, had an illuminance of 60 lux of full-spectrum fluorescent light, and the air temperature fluctuated between 23.5 and 29.5 °C. The incubator dawn was 8 am (to allow earlier egg laying for other experiments), had an illuminance of 2000 lux of full-spectrum fluorescent light, and the air temperature was tightly regulated at 28.5 °C. Because the water temperature was not regulated within the spawning tanks, the room temperature affected the water temperature. Spawning tanks were left undisturbed overnight on a designated vacant shelf of the standard housing rack or the fry incubator. The total information collected for each spawning tank was the set-up date, day of the week, tank ID, number of males and females, spawning room, and presence or absence of each: dividers, plants, and marbles.

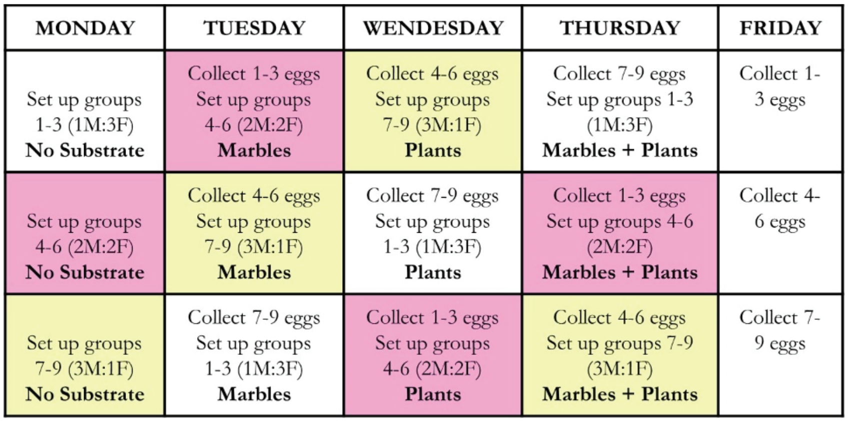
In the morning at lights on (dawn), spawning tank water was replaced with fresh system water in tanks without dividers. In the incubator, fresh system water was prefilled the night before so the water change would be a consistent temperature. At the same time, dividers were removed from divided tanks and water was not replaced to maintain the pheromone cues. Approximately two hours after dawn, spawning tanks were checked for eggs. Clutches were collected, counted, and recorded. All fish were returned to their home tanks before the first feeding.

Two sets of experiments were conducted: a retrospective analysis of spawning records to identify patterns and controlled breeding events to test those patterns.

Retrospective spawning analytics

Groups of two to seven wild-type fish were set up to spawn from over a span of two years for routine laboratory use. The previous reproductive success of the fish varied from first controlled spawning to experienced breeders and included fish born and raised in-house and fish acquired externally. The variable spawning parameters were at the discretion of whomever of the authors set up the spawning tanks that day. These variable parameters were the spawning location, the number and sex ratio of fish, and tank additions (dividers, marbles, and aquarium plants). The divider was used to separate male and female fish before dawn. Dividers were added at the time of spawning tank set up, before fish were added. Water could flow freely between the two sides of the divided tanks below the level of the slotted tank liner. Ratios of fish were grouped into equal sex, male-dominant, and female-dominant groups. Equal sex groups included groupings of two or four fish. Male-dominant groups included ratios of 2:1, 3:1, 3:2, 4:1, and 4:3 of males to females. Female-dominant groups included ratios of 1:2, 1:3, 1:5, and 2:3 of males to females. Reproductive success as a function of age and of season were also measured. No other persons handled these fish for the duration of these experiments.

These analyses pooled data from several home tanks ("colony" in figures) to assess the aggregate effects of the various parameters. The collected data from a single home tank ("1 tank" in figures) of nine fish (four females, five males; date of birth 3-28-17) from August 2017 to March 2019 was used to assess how these manipulations affect the breeding behavior of the same fish over time. These results were compared to the colony data in some of the analyses as indicated in each section or figure. Because the variables tested occur together and may influence reproductive success in complex ways, individual factors and compound effects were compared. First, each independent variable was collapsed into a binary (presence or absence; group or pair; room or incubator) or three to four variate analyses (age bin, season, group bin) to assess the contribution of each variable on a broad scale. Following the binary analysis, interactions among variables were assessed by grouping between two attributes at a time, such as marbles and room, to generate assessments between four groups: marbles + room; marbles + incubator; no marbles + room; no marbles + incubator.



MONDAY	TUESDAY	WENDESDAY	THURSDAY	FRIDAY
Set up groups 1-3 (1M:3F) **No Substrate**	Collect 1-3 eggs Set up groups 4-6 (2M:2F) **Marbles**	Collect 4-6 eggs Set up groups 7-9 (3M:1F) **Plants**	Collect 7-9 eggs Set up groups 1-3 (1M:3F) **Marbles + Plants**	Collect 1-3 eggs
Set up groups 4-6 (2M:2F) **No Substrate**	Collect 4-6 eggs Set up groups 7-9 (3M:1F) **Marbles**	Collect 7-9 eggs Set up groups 1-3 (1M:3F) **Plants**	Collect 1-3 eggs Set up groups 4-6 (2M:2F) **Marbles + Plants**	Collect 4-6 eggs
Set up groups 7-9 (3M:1F) **No Substrate**	Collect 7-9 eggs Set up groups 1-3 (1M:3F) **Marbles**	Collect 1-3 eggs Set up groups 4-6 (2M:2F) **Plants**	Collect 4-6 eggs Set up groups 7-9 (3M:1F) **Marbles + Plants**	Collect 7-9 eggs

Figure 1. Seasonal spawning rotations. Groups of four sexually mature, laboratory-raised fish from mixed parentage were set up in triplicate (three sex ratios, nine total tanks) with three sex ratios: one male (M) and three females (F) (1M:3F), 2M:2F, or 3M:1F in a rotating cycle. Spawning substrate was consistent to the day of the week. This three-week setup was repeated three consecutive times for a total of twelve breeding events per tank.

Controlled spawning environments

Groups of four sexually mature (three to six months old), in-house raised fish from mixed parentage and a minimum of two generations removed from externally-sourced founder fish were set up in triplicate (three sex ratios, nine total tanks) with different sex ratios (3M:1F, 2M:2F, or 1M:3F). These groups were set up to spawn on a rotating schedule for three months (spanning a season) according to the following rotation: all three tanks from a sex ratio group were set up to spawn rotating through four consecutive days each week (**Figure 1**). Thus, one sex ratio group would spawn on days one and four. The substrate was kept the same each week such that the spawning tanks set up on day one had no substrate, day two had marbles, day three had plants, and day four tanks had both marbles and plants. Thus, each sex ratio grouping spawned in each substrate condition three times and on each day of the week three times for a total of twelve spawning events during the experiment. This setup was repeated each season with a new batch of fish so that the fish were the same age at the start of each season. Seasons were defined as follows: January 1–March 31 as winter, April 1–June 30 as spring, July 1–September 31 as summer, and October 1–December 31 as fall. These trials were all conducted in the standard fish housing room with no dividers.

Reproductive success

Reproductive success was assessed in two ways: clutch success and the number of eggs per female. Clutch success was defined as the presence of at least one egg in the spawning tank at the time of collection (roughly two hours after dawn) and was scored as either zero (no eggs) or one (eggs), thus generating a probability of egg generation. Egg viability was not assessed. Tanks with multiple females were scored as a single clutch for determining clutch probability as a function of the number of animals in the group. The number of eggs per female is the total number of eggs in the spawning tank divided by the number of females in that tank. This value was counted as multiple, equal sized clutches to assess the average number of eggs per female.

Statistical analyses

Values are expressed as means (M) ± the standard error of the mean (SEM). Unpaired two-tailed t-tests were used to compare means between two groups. Comparisons between observed and expected means were compared using two-tailed one-sample t-tests. Analyses between three or more groups were made using one-way ANOVA with post-hoc Tukey's multiple comparisons test. Multivariate analyses with two-way ANOVA followed by Tukey's multiple comparisons tests were used to test variable interactions. P < 0.05 were considered significant. GraphPad Prism 8 software (San Diego, CA) and Microsoft Excel were used for analyses and graphs.

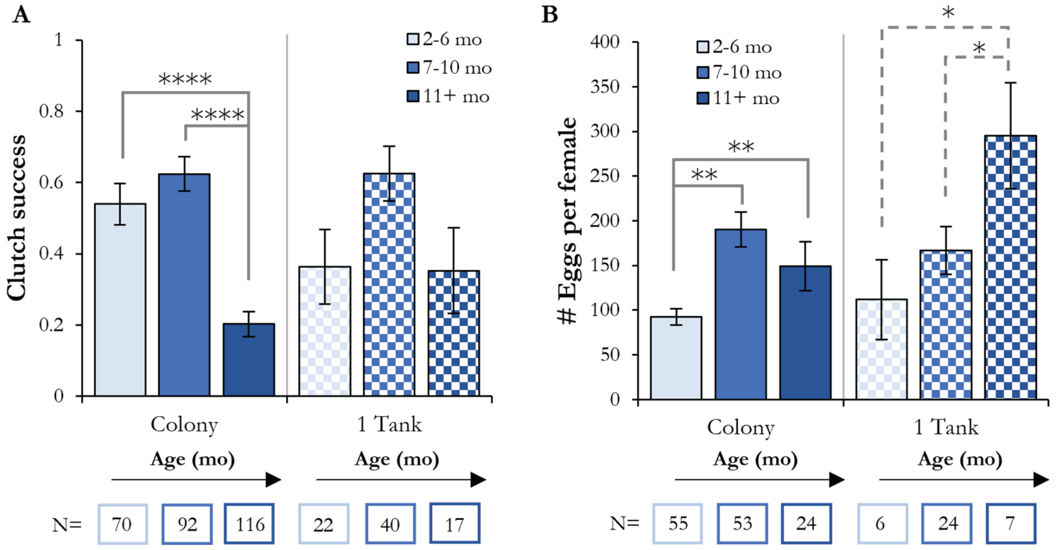


Figure 2. Zebrafish function as an annual species. Spawning success and clutch size declined after 10 months (mo) of age in most of the colony. **A.** Clutch success was similar in young (2–6 mo) versus adult fish (7–10 mo) and was reduced in aged fish (11+ mo). The single tank (checkered bars) shows the behavior of the same fish across all ages examined versus the colony. **B.** Adult fish produced the largest clutches in the colony and aging adults produced even larger clutches in the single tank. Values depict mean ± SEM. N = number of spawning events, indicated under each bar. Statistical significance tested by one-way ANOVA with Tukey's multiple comparisons test. * p < 0.05, ** p < 0.01, **** p < 0.0001.

RESULTS

Age

To test the suggestion that young and aged fish reproduce less successfully than mature adults, the retrospective data was binned into one of three age categories: young (2–6 months), adult (7–10 months), and aged (11+ months). Age had a significant effect on clutch success [F (2, 281) = 29.59, p < 0.0001] (**Figure 2A**) and clutch size [F (2, 129) = 9.578, p = 0.0001] (**Figure 2B**). Aged fish produced significantly fewer clutches (M = 0.16 ± 0.03) than young fish (M = 0.54 ± 0.06) or adults (M = 0.61 ± 0.05). Though clutch success was comparable between young fish and adults, adults produced larger clutches (M = 197.3 ± 21.89, p < 0.0001) than either young (M = 90.00 ± 9.31) or aged fish (M = 135.1 ± 30.75). Analysis of a single tank of fish over the course of one year highlighted the consistency of the pattern of clutch success (**Figure 2A**) and revealed that these experienced breeders who were spawned routinely produced significantly larger clutches (M = 295.1 ± 59.58) as they aged [F (2, 34) = 3.484; p = 0.042] (**Figure 2B**). Peak clutch probability and size occurred at eight months of age (data not shown).

Fish groupings

Clutch probability and size were compared between male-female pairs, female-dominant groups, male-dominant groups, and equal sex groups (**Figure 3A**). Clutch probability was significantly greater for groups (group M = 0.60 ± 0.05) versus pairs (M = 0.35 ± 0.03; $p < 0.0001$; **Figure 3B**). Significant differences in clutch success emerged between male-dominant, equal sex breeding (equal sex groups and pairs combined), and female-dominant groupings [F (2, 269) = 7.366, p = 0.0008]. Male-dominant groups had significantly greater clutch probability (M = 0.66 ± 0.07) than equal sex groups (M = 0.37, ± 0.03; p = 0.0006; **Figure 3C**). The number of eggs per clutch was comparable between groups and pairs (group M = 149.8 ± 16.57 versus pairs 131.4 ± 14.45, $p > 0.05$) when controlled for the number of females present (**Figure 3D**). Clutch sizes by sex ratio followed a similar pattern as clutch success, with the largest clutches occurring in male-dominant groupings [F (2, 135) = 3.512; p = 0.0326] (**Figure 3D**). Data from the single tank revealed contradictory results. Pairs and groups produced clutches with equal frequency (**Figure 3B**) while females produced more eggs in a group setting (group M = 319.4 ± 54.33 versus pairs 150.1 ± 22.68, p = 0.0033; **Figure 3D**). For the colony as a whole, groups of fish with males outnumbering females had the highest reproductive success by both measures of success.

The effect of sex ratios was tested with groups of four (**Figure 3A**). Clutch success was highest in equal sex groupings (**Figure 3C**; M = 0.52 ± 0.03) [F (2, 584) = 5.819; p = 0.0295]. Females produced the largest clutches when outnumbered by males (**Figure 3E**; M = 118.2 ± 13.65) [F (2, 485) = 26.23; $p < 0.0001$].

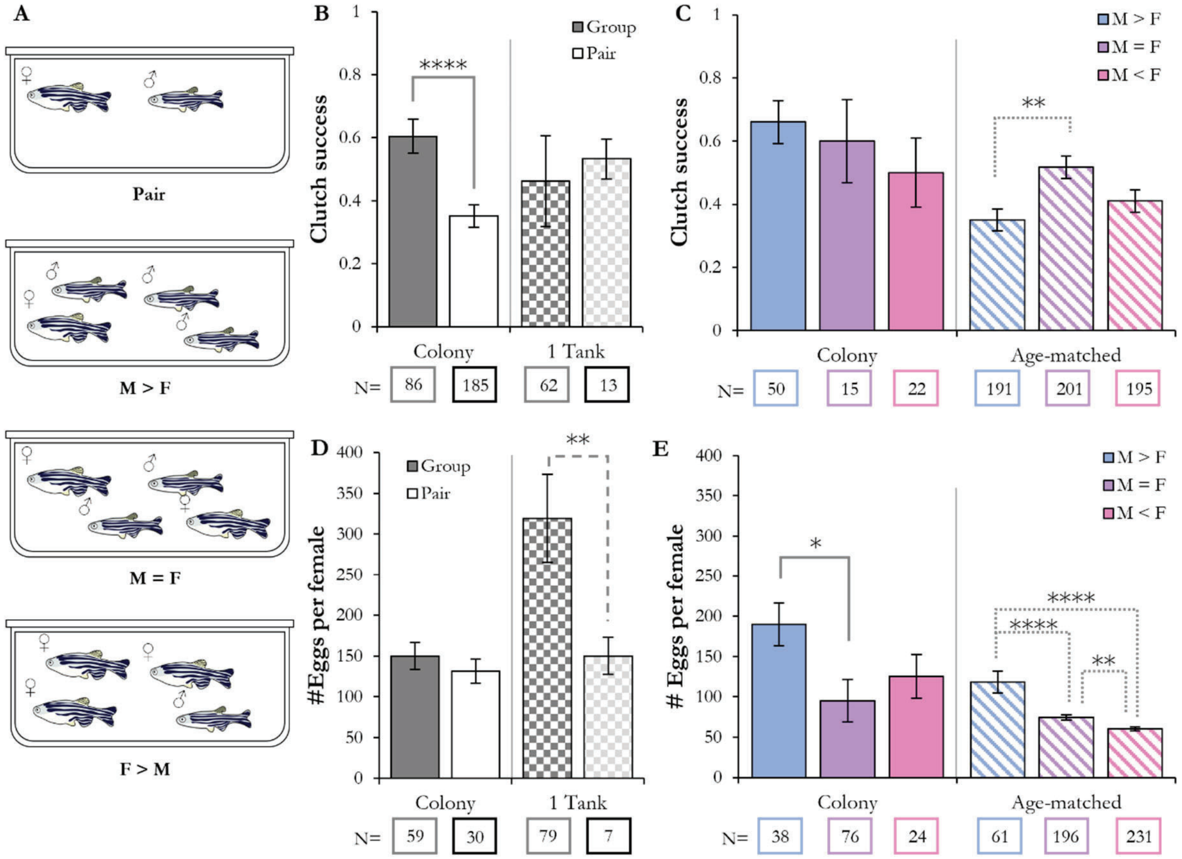


Figure 3. Male-dominant groupings enhance reproductive success. **A.** Pairs, equal sex (M = F), male-dominant (M > F), and female-dominant groupings (M < F) were compared from all fish (colony), a single tank (checkered bars), and age-matched groups (striped bars). **B.** Groups of fish had higher clutch success than pairs. **C.** Equal sex groups had highest clutch success in age-matched fish, but not in the colony as a whole. **D.** For most of the colony, females lay equal numbers of eggs in pair or group settings. However, the fish in this sample tank produced more eggs in group settings. **E.** Females produced larger clutches when outnumbered by males in the colony and in age-matched tanks. Values depict mean ± SEM. N = number of spawning events, indicated under each bar. Statistical significance tested by unpaired t-tests (B/D) or one-way ANOVA with Tukey's multiple comparisons test (C/E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Tank additions

Dividers separated the sexes overnight, a single plastic plant was used to provide shelter and hiding places, and marbles were meant to mimic spawning substrates like rocks (**Figure 4A**). From the retrospective analysis, the use of dividers did not affect clutch probability or size (**Figure 4B**). Clutch size and egg probability were compared between tanks with no substrate, or any substrate (a plant, marbles, or both plant and marbles). The clutch probability and size were not significantly different. When comparing each habitat substitute separately, clutch probability and size between these groups were not statistically significant (**Figures 4C** and **4D**). However, the presence of both marbles and plants significantly increased clutch success [(F (3, 305) = 3.701; p = 0.0121].

In age-matched groups, clutch probability was comparable between substrate conditions [F (3, 865) = 1.173; p = 0.3188, not significantly different, **Figure 4C**], while the clutch size differed between conditions [F (3, 479 = 5.674, p = 0.0008]. Fish spawned with both marbles and plants produced the largest clutches (**Figure 4D**; M = 87.06 ± 7.879) and without any substrate produced the smallest clutches (M = 58.05 ± 7.879). The use of any type of substrate, versus none, was associated with significantly larger clutches (p = 0.0007 **Figure 4D**).

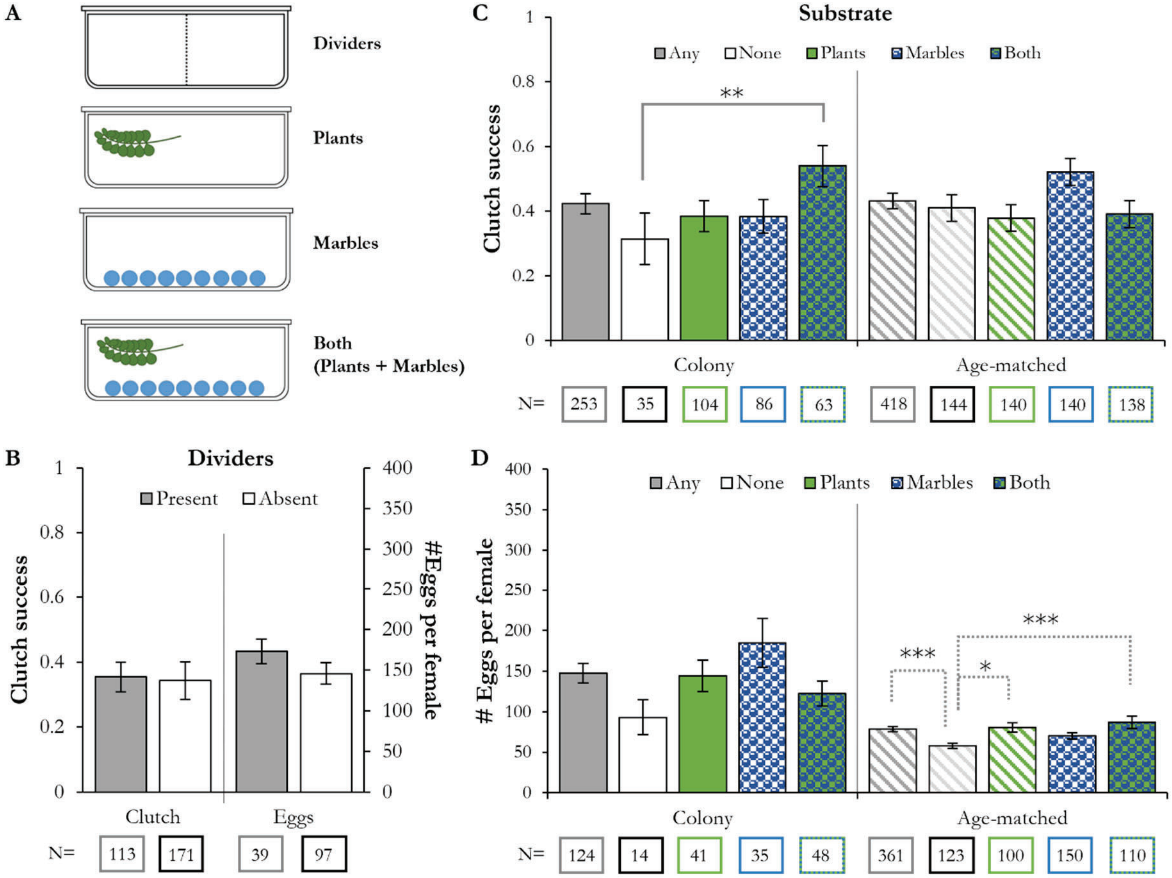


Figure 4. A habitat substitute increases reproductive success. **A.** Tank additions include clear dividers, plants, and marbles. **B.** The use of dividers had no effect on clutch probability or size. **C.** Presence versus absence of any substrate (plants, marbles, or both) did not significantly affect the probability of clutch success. Marbles + plants offered additional benefit to increase clutch success in the colony but not the age-controlled experimental groups (striped bars). **D.** Type of substrate had no significant impact on the number of eggs per female in the colony at large, while age-matched fish produced slightly larger clutches with marbles and/or plants. Values depict mean ± SEM. N = number of spawning events, indicated under each bar. Statistical significance tested by unpaired t-tests (B) or one-way ANOVA with Tukey's multiple comparisons test (C/D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Spawning room parameters

Spawning tanks were placed on a shelf in either the standard housing room or the fry incubator (**Figure 5A**). Egg probability and clutch sizes between groups spawned in the incubator and housing room were not statistically significant, though there was a trend toward larger clutches in the housing room (p = 0.06; **Figure 5B**). While each tank substrate (plants, marbles) independently did not affect spawning success, nor did the spawning location, the room and tank additions mattered depending on the combination of these factors. Fish were more likely to lay eggs in the housing room if plants were present (M = 0.48 ± 0.05) and more likely to spawn in the incubator if plants were absent (M = 0.46 ± 0.07) [F (1, 276) = 5.49; p = 0.0198] (**Figure**

5C). Clutches were of comparable size in these conditions. Reproductive success in the presence of marbles did not show this pattern.

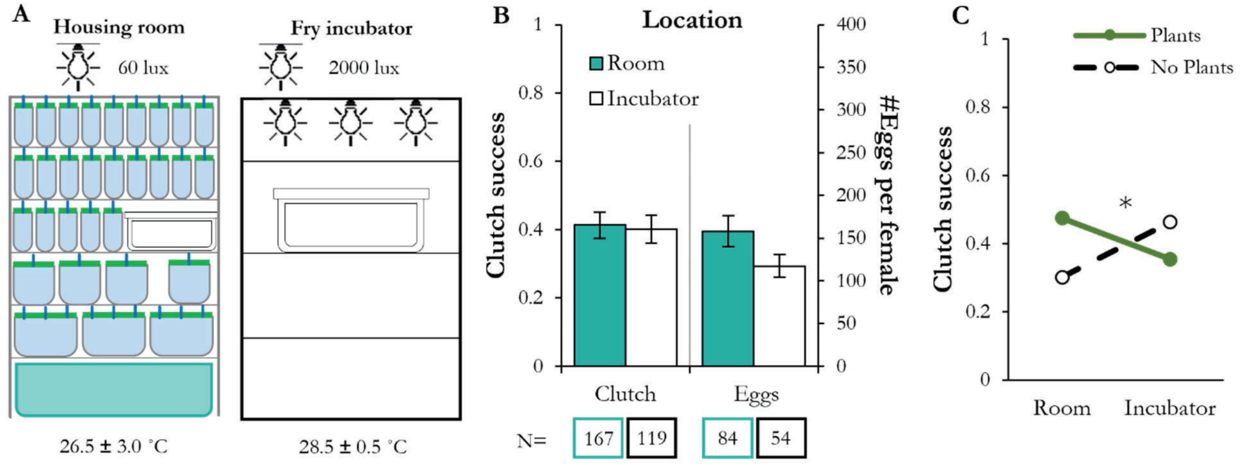


Figure 5. The spawning location does not affect reproductive success. **A.** The standard housing room and fry incubator have different temperature and lighting conditions. **B.** Fish spawned in the room and incubator had equal spawning success and clutch sizes. **C.** Plants best predicted spawning success in the standard housing room. Values depict mean ± SEM. N = number of spawning events, indicated under each bar. Statistical significance tested by unpaired t-tests (B) and two-way ANOVA with Tukey's multiple comparisons tests (C). * $p < 0.05$.

Seasonal effect

From the retrospective analysis, there was a significant effect of season on breeding success of laboratory-raised zebrafish [F (3, 289) = 6.297; p = 0.0004]. Spawning in the winter (Jan–Mar) or spring (Apr–Jun) was associated with higher clutch probability (winter M = 0.49 ± 0.05; spring M = 0.65 ± 0.08) and summer (Jul–Sep) spawning had the lowest clutch success (M = 0.31 ± 0.05) (**Figure 6A**). Differences in clutch size followed the same pattern [F (3, 138) = 4.035; p = 0.0087] (**Figure 6B**), with largest clutches in the spring (M = 186.9 ± 31.62). Analysis of the single tank of fish over the course of one year followed similar trends as the larger colony. This particular tank produced significantly more eggs in the spring [F (3,33) = 2.987; p = 0.0451], despite being older than ten months at the time. Compared to the clutch probability of fish in this age group, these fish had significantly higher clutch success (p = 0.04). Reproductive success was highest when fish were spawned in the spring and poorest in the summer. When controlled for age reproductive success was poorest by both measures in the fall, with fewer (M = 0.11 ± 0.03 [F (3, 576) = 33.52; p < 0.0001, **Figure 6A**] and smaller clutches (M = 49.72 ± 4.723) [F (3, 484) = 8.523; p < 0.0001, **Figure 6B**].

DISCUSSION

Zebrafish gained popularity as an experimental model in part because they breed well in captivity. Even so, zebrafish reproduction is incredibly variable between individual fish, genetic lines and strains, and institutions. Spawning success in zebrafish requires knowledge and some intuition that many fish facility staff or researchers do not have. Because of this, the techniques used at different institutions have created confusion regarding best spawning practices. This set of experiments retrospectively analyzed and subsequently tested published and anecdotal best practices for laboratory-housed zebrafish spawning success through correlative analysis over two years and subsequent manipulations. These results should guide zebrafish users to maximize spawning success. The probability of clutch success (presence or absence of eggs) and the number of eggs per female were compared with the following tracked or manipulated variables: age, sex ratios, groupings, habitat substitutes, dividers, light intensity, temperature fluctuations, and seasons. While some of these results support published recommendations, there are several instances that are in direct contrast to such recommendations.

Previous publications reported that adults between the ages of 7–12 months lay the most eggs[3, 7] Consistent with previous published reports, the probability of clutch success and the size of individual clutches were highest among fish aged seven to ten months, though with an exception: consistent breeders continue to produce frequent, large clutches well beyond ten months. The current study found the most frequent and largest clutches were produced by eight-month-old fish. This coincides with the suggestion that while zebrafish can live and reproduce well beyond one year, in reality they function as an annual species.[30] While the benefits of spawning 7–10 month-old fish is clear, it is encouraging that fish as old as 25 months are capable of spawning successfully because this expands the timeframe that fish may be useful, particularly among transgenic fish that may otherwise be precious and maintained in a colony longer than would their wild-type counterparts. Furthermore, highly fecund fish that are spawned regularly continued to produce large clutches beyond ten months of age, suggesting that consistent breeders, which are known to have greater reproductive success[3] need not be retired until fertility declines. One limitation to the interpretation of the current data, particularly with respect to aging, is that the present analysis did not track viability of eggs or survival of larvae. As such, egg quality may indeed vary with the age of the parents as some have previously suggested, at least in young parents.[3]

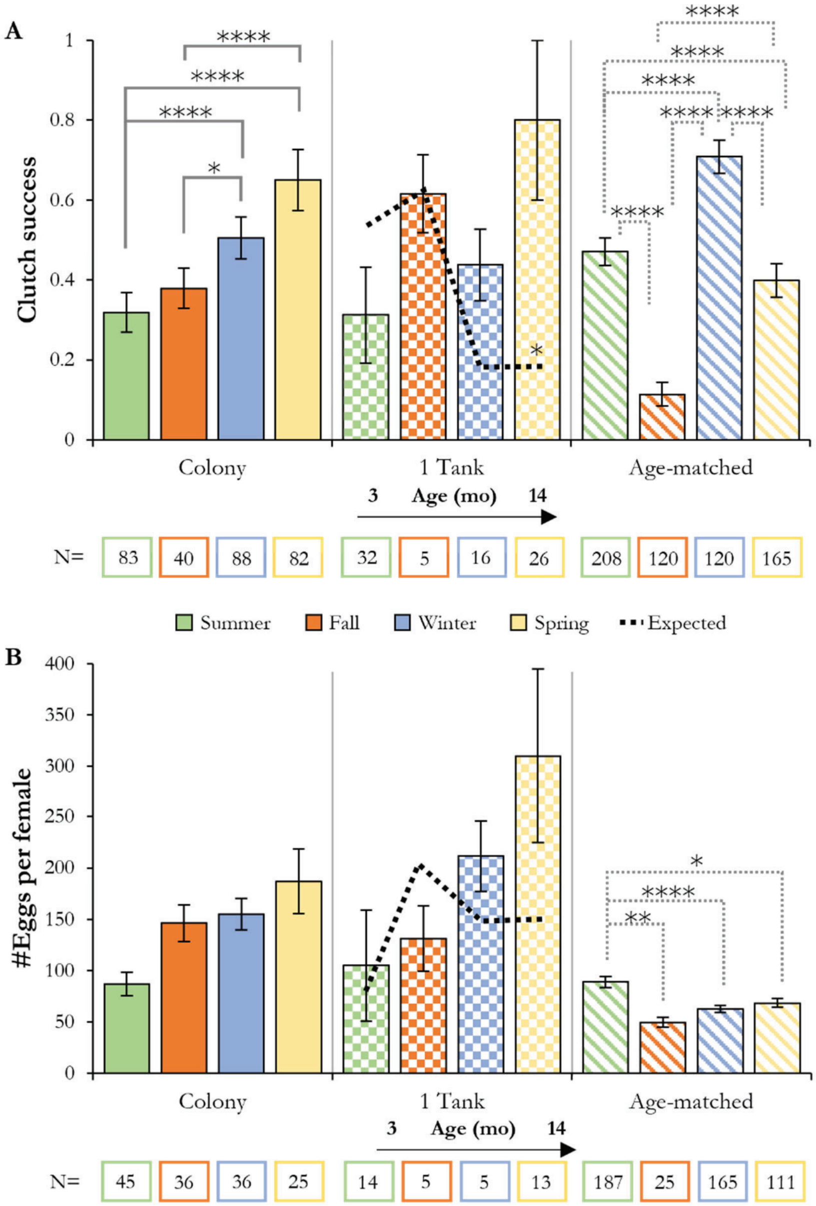


Figure 6. Laboratory-housed zebrafish exhibit seasonal spawning patterns. **A.** Zebrafish clutch success was highest in the spring (April–June, yellow) and lowest in the summer (July–September, green) for both the colony and a single tank (checkered bars). The age-matched controls (striped bars) had higher clutch success in winter (January–March, blue). **B.** Clutch size followed the same pattern, with the largest clutches in spring. A single tank of fish bred over an entire year followed the same trends. These patterns did not match predicted reproductive success based on the age of the single tank (dotted line, * on bar). The age-matched fish produced smaller clutches overall, but with the largest in summer. Values depict mean ± SEM. N = number of spawning events, indicated under each bar. Statistical significance tested by one-way ANOVA with Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$.**** $p < 0.0001$.

While the age-related decline in fertility is clear, it is expected that the apparent sharp decline in fertility after ten months is in reality more gradual, as the current dataset did not have spawning data for every month of age after ten months, but rather binned by ages into young, adult, and aged fish. Fish as young as two months old successfully reproduced. Because the age at which zebrafish reach sexual maturity spans a range of months[7, 14] size is a better indicator of sexual maturity. Precise assessment of the contribution of size to spawning success can be stressful for the fish because it requires significant handling[31] and is likely unnecessary for routine spawning. Because size has been previously linked to reproductive success,[8, 10, 32] the current report assessed raw age independent of size. These data support spawning fish once males and females can be visually distinguished, regardless of the exact age.

Laboratory-raised fish are often spawned in groups.[7, 14, 15] Previous data suggested highest egg success would be achieved with female-dominant groupings (more females than males).[7, 12, 18] The present data suggests rather the opposite - females were more likely to lay eggs when outnumbered by males or when not competing with another female. Some groups have reported male-dominant large groups generate more eggs because it ensures all females have access to a mate,[20] which the present data supports. Spawning fish in groups, as is common in laboratory settings, led to higher clutch probability, but not larger clutches than pair-spawning. This suggests two possibilities: females may lay fewer eggs in a group setting, or not all females are laying eggs in these groups. Without directly assessing parentage, the latter scenario is likely, given that our data indicate that the clutch size is consistent between all groups of four fish, regardless of sex ratio. The previous reports that females prefer to spawn with a favorite male[32] and that wild fish spawn in pairs[13] suggests the primary benefit of group spawning is an increased likelihood of matching a preferred spawning pair together. Therefore, spawning fish in groups from the same home tank best ensures reproductive success by allowing females to choose their preferred mate, which could also be one reason why clutch success was greater in male-dominant groups – the single female was more likely to be paired with her preferred mate without any same sex competitors. Recommended spawning practice is therefore to set up groups of two males and one female.

The greater success of male-dominant groups also suggests that the presence of multiple females may actually decrease egg production, possibly due to intrasex conflict. This finding is in direct contrast to published recommended spawning practices promoting female-dominant groups[7, 14, 18] that perhaps did not take female choice preference and female-female competition into account. Males and females both initiate intrasex aggression,[33, 34] exhibiting such behaviors as chasing, circling, and biting. Because chasing and circling are exhibited during mating displays between males and females,[35, 36] the present study did not specifically assess these behaviors. Quantifying intrasex competition in spawning tanks may correlate with reproductive success in group spawning. While group spawning was associated with greater clutch probability, it is important to note that pairs laid similar sized clutches as group breeders because laboratory experiments often require controlling for the parental genotype, often necessitating pair breeding. The current results suggest that successful pairs lay equally large clutches as group breeders. Pairs that fail to breed may benefit most from simply trading for a different mate.

Many researchers spawn with clear plastic dividers to segregate males and females overnight in an effort to increase spawning success.[4, 19, 20] Dividers separate males and females while allowing them to detect pheromones and visual cues (**Figure 4A**). When spawning pairs of zebrafish, some researchers recommended segregating the fish using clear plastic dividers to allow fish to observe visual and olfactory cues prior to mating.[21, 22] The practice of using dividers in the spawning tank is purported to increase reproductive success[4] and to allow more controlled timing of egg laying.[19, 20] The present study found no significant differences in clutch probability or size with the use of dividers. Segregating the sexes within the spawning tank is time-consuming and the current findings are important not only because it suggests segregating fish before dawn offers no benefit in the frequency of spawning events or number of eggs, but also because the use of dividers did not hinder reproductive success. Therefore, the use of dividers that are removed when eggs are desired can allow more controlled timing of breeding without significantly impacting egg production.

Perhaps one of the most varied parameters in zebrafish spawning between institutions and users is the use of habitat substitutes in the spawning tank.[7, 12, 27, 37] The present results suggest that fish are more likely to spawn in the presence of some sort of habitat substitute, and the use of both plants and marbles maximized clutch size.

At times it is necessary to change the spawning environment. Fish may be transferred to different breeding rooms after quarantine, or may be transferred to a housing system on a shifted light cycle to generate eggs at a different time of day. This experiment used two spawning rooms to estimate the impact these types of scenarios may have on reproductive success. The fry incubator has a higher, more stable temperature, higher daytime luminescence, and fewer sources of potential light pollution than does the standard housing room. The housing room has more potential sources of light pollution (door gaps, monitoring system LEDs) that may negatively affect reproductive success.[25] There were no significant differences in clutch probability or size between these two rooms, suggesting that fish have a wide range of tolerance for temperature fluctuations and light intensity, and these factors do not significantly influence spawning success. These results are limited in that it is not possible from the current dataset to distinguish the contributions of each of these variables individually. Air (and therefore tank water) temperature fluctuations during the day or across seasons, as well as light levels, could potentially affect reproductive success independently.

However, the fact that the fish show wide ranges of tolerance for temperature fluctuation, light level, and basic room differences is encouraging because it means that zebrafish can be transferred to different housing rooms or light cycles as is experimentally relevant with minimal impact on reproductive success. The spawning location did affect fish behavior in that fish exhibited a preference for plants when spawning in the standard housing room. While the reason for this preference is unclear, the difference in illumination of the two locations may play a role. Fish behavior may be different in the spawning environments due to stress and differences in social interactions. In the incubator, where light is brighter and the water is warmer, fish may be more vigilant for predators and engage in fewer intraspecific conflicts, while in the housing room, fish are using the plants as hiding places to escape from aggressive mates or as territories to be guarded.[38] Further experiments would be necessary to test these possibilities.

Anecdotally, zebrafish researchers often experience a spawning slump during the summer months and this was the impetus for the present study. Laboratory-housed fish are kept year-round on a 14:10 h light:dark cycle to mimic their natural spawning season to generate eggs year-round,[7, 13] and fed a standard diet, eliminating seasonal differences in prey that have been suggested as a source of seasonal spawning differences.[15] Water quality and temperature are consistent year-round. In spite of these controls, there was a strong effect of season on laboratory-housed zebrafish spawning. Fish produced more frequent, larger clutches in spring. Importantly, this seasonality trend does not appear to be correlated with the ages of the fish. Using age-related spawning data from both previous data[3, 7] and results from this paper, the expected seasonal output of the single tank of fish was expected to be highest between fall and winter, when these fish were 6–11 months old. Instead, their reproductive output was highest in the spring when they were 12–14 months old (**Figure 6**). Because these lab-raised fish are kept on a consistent light cycle, water temperature, and nutrition source, the fish may be detecting some other factor of seasonality that is not being controlled in the laboratory environment. This raises the question, what are they detecting? One possibility is barometric pressure. Rapid drops in barometric pressure, as occur preceding precipitation events, changes the behavior of wild fish and sharks,[39–41] which could affect reproductive behavior. Regardless of the underlying cause, this seasonal breeding component should be kept in consideration for experiments requiring frequent clutches. Experiments that require frequent clutches of eggs would be best suited for winter or spring months whenever possible, and setting up extra spawning tanks during the summer and fall may be necessary to generate sufficient eggs.

CONCLUSIONS

Studies researching zebrafish spawning practices in a laboratory setting are important because of the magnitude of zebrafish use in science. It is also important to study spawning in order to maximize spawning events and egg count for research in which zebrafish are used as a model. The optimal conditions were groups of three fish (two males and one female) aged seven to ten months spawned in the spring with both plants and marbles in the spawning tank. These data support the implementation of simple steps that reliably maximize reproductive success of laboratory zebrafish.

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PRESS SUMMARY

Zebrafish are common biological model organisms that are bred in laboratory settings. This study identified conditions that promote maximum clutch probability and size in laboratory zebrafish. These data support the implementation of simple steps that reliably maximize reproductive success of laboratory zebrafish while raising some interesting questions about how zebrafish exhibit seasonal behaviors in a climate-controlled environment.