Investigating the effects of algal turbidity on mate choice and reproduction of the dwarf seahorse (*Hippocampus zosterae*)

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ABSTRACT:

Algal-induced turbidity can alter several important aspects of reproduction and sexual selection. The dwarf seahorse (*Hippocampus zosterae*) is a monogamous species that displays reciprocal courtship behaviors, exhibits male pregnancy, and serve as an indicator species for threatened seagrass ecosystems. Given the year-round variation in turbidity reported at our study site in Tampa Bay, Florida, this study investigated the impact of algal turbidity and varying sex ratios on mate choice parameters and reproductive success of seahorses. The experimental design consisted of an equal 1:1 sex ratio and two sex-biased treatments: female-biased (2F:1M), and male-biased (1F:2M) with varying sized mate options in clear and turbid water (n = 8, 128 seahorses). In the turbid treatment, mating latency was significantly lower, and the number of non-transferred eggs is significantly higher than for fishes in clear water. However, the mate choice preferences, reproductive success, and behavioral rates were not impacted due to turbidity. Results reveal that behavioral investment is a strong indicator of mate choice preferences in sex-biased treatments regardless of turbidity. The reproductive output was greater in the sex-biased treatments as compared to the paired treatments. These minimal impacts of turbidity suggest that dwarf seahorses may have adapted to the varying seasonal eutrophic conditions in Tampa Bay and provide validity for prior findings from laboratory behavioral studies in this species. The findings of this study will allow us to predict potential changes in the mating system and reproductive success of seahorses under threat from higher fluctuation of human induced eutrophic conditions.

INTRODUCTION:

Anthropogenic environmental changes pose a significant threat to the persistence of animal populations globally. The alarming acceleration of these human-driven actions causes habitat destruction, pollution, overfishing, ocean warming and acidification, and climate change (Coimbra et al., 2020; Gao et al., 2020; Harasti, 2016; Jackson et al., 2001; Prabhakar et al., 2012; Trenberth, 2018). This impacts the ecological structure, population survival, physiological and behavioral traits of organisms (Chown et al., 2010; Lane et al., 2011; Nabe-Nielsen et al., 2018, Tuomainen & Candolin, 2011).

The human-induced enhancement of nutrients in aquatic ecosystems, particularly nitrogen and phosphorus lead to excessive algal blooms causing eutrophication (Smith et al., 2006, Ansari & Gill, 2014). The introduction of excess nutrients can disrupt the ecosystem's equilibrium and pose a threat to the aquatic ecosystem. Mainly, excess growth of algae creates high levels of turbidity in the water column, hindering light availability underwater (Anderson et al., 2002). A recent example of this is the Piney Point wastewater release in March-April 2021, where approximately 814 million liters of phosphate mining wastewater was released into the lower part of Tampa Bay, Florida due to a failing gypstack. This resulted in an accumulation of 186 metric tons of nitrogen, a limiting nutrient in the bay (with ammonium concentration of 200mg/L), alongside estimated addition of 50-100 tons of phosphorus (Major & Pasek, 2021). These nutrient influxes, particularly nitrogen, triggered exponential growth of cyanobacteria (*Dapis* spp.) and red tide (*Karenia brevis*) populations, leading to a turbid environment (Beck et al., 2022, 2023; Greening and Janicki, 2006; Greening et al., 2014; Major & Pasek, 2021;

Wang et al., 1999). These surplus nutrients caused an increase in algal blooms and turbidity, which decreases the clarity of the water (Anderson et al., 2002).

Previous studies on turbidity have reported mixed effects on aquatic organisms. Coastal fishes in turbid water have been shown to have decreased numbers of eggs, a lack of parental care, and hampered predator avoidance (Lane et al., 2011). In eutrophic conditions, with limited sunlight availability, guppies (*Poecilia reticulata*) exhibit solitary behavior, with conspicuous coloration but reduced courtship activity (Borner et al., 2015; Camargo-dos-Santos et al., 2021). Increased water turbidity impairs chromatic vision in Lake Victoria cichlids, favoring selection for less fecund, dull morphs over brightly colored conspecifics unlike in clear environments. Moreover, turbidity reduces female selectivity for male coloration. (Maan et al., 2010; Seehausen et al., 1997). In sand gobies (*Pomatoschistus minutus*), inability for females to perceive the competition from other females leads to reduced clutch of eggs on the male's nest. However, the survival rate of eggs is increased in turbid water with decreased parental care (Järvenpää et al., 2019; Järvenpää & Lindström, 2011).

Turbidity enhanced sexual selection on male courtship behaviors for mate attraction in threespine stickleback (*Gasterosteus aculeatus*). However, regardless of turbidity, final spawning decision by females was not impacted by the courtship displays but mediated by olfactory cues related to Major Histocompatibility Complex class IIB (MHCIIB alleles) (Bahr et al., 2012; Engstrom-Ost & Candolin, 2007; Heuschele et al., 2009; Milinski, M., 2006; Milinski et al. 2005; Reusch et al. 2001). An increase in algal density enhanced the reproductive cycles and increasing hatching success rates of male sticklebacks (Candolin et al., 2016). Turbidity also enhances critical swimming performance in Golden Shiners (*Notemigonus crysoleucas*) (Hildebrandt & Parsons, 2016). In the African cichlid, (*Pseudocrenilabrus multicolor victoriae*), turbidity reduced overall activity level and social behaviors, but elevated levels of aggressive behaviors were noted (Gray et al., 2012).

As turbidity has been correlated with obstructing visual cues in the water column, it could alter mating behaviors, social behaviors, and reproductive success of an organism. In one study where turbid waters were simulated by reducing visual cues, polygynandrous male broad-nosed pipefish (*Syngnathus typhle*) were unable to discriminate between sexes when using only olfactory cues, unlike the turbidity-adapted males of the gulf pipefish (*Syngnathus scovelli*) (Lindqvist et al., 2011; Ratterman et al., 2009). Male broad-nosed pipefish spent less time accessing their mate, which may lead to increasing the mate search time (Lindqvist et al., 2011; Sundin et al., 2010). Regardless, male broad-nosed pipefish used olfactory cues mediated by Major Histocompatibility Complex class I genes (MHC) in the turbid waters for mate selection, favoring females with dissimilar MHC genotypes. This shift from using visual to olfactory cues potentially enhances offspring fitness, compared to selection based solely on body size (Roth et al., 2014). Sundin et al. investigated the role of turbidity in broad-nosed pipefish (*Sygnathus typhle*) by simulating turbidity hampering visual and/or olfactory cues (2010) and by simulating turbid condition in water column and allowing free interactions (2017). The results from former studies suggested lower sexual selection, however in the later experiment, the social environment in turbid environment allowed inter-individual interaction, promoting competition and selective mate choice, which increased mating and reproductive success. It also enhanced sexual selection on male length (Sundin et al., 2010, 2017). In polygynandrous straight-nosed pipefish (*Nerophis ophidion*), high sexual selection observed for female ornamentation in clear waters. However, in the turbid environment, perception of sexual signals weakened, relaxing the sexual pressure on female ornamentation, leading to randomized mating (Sundin et al., 2015).

Animal behaviors serve as reliable indicators of overall health and can also be used to transmit important information to conspecifics. Deviations from typical behaviors can signal underlying physiological or environmental stressors. Seahorses have elaborate courtship rituals with series of behaviors that occur prior to the females' transferring eggs into the male's brood pouch (Rosenqvist and Berglund, 2011). In the monogamous Australian seahorse (*Hippocampus whitei*) and the Sri Lankan seahorse (*Hippocampus fuscus*), the mating pair perform morning rituals by reciprocal quivering with tails grabbed together at the dawn. This allows the pair to establish and maintain the pair bond and strong sexual fidelity (Vincent, 1995; Vincent & Sadler, 1994). Mate quality plays a crucial role in determining reproductive success and fitness of an organism. The pattern of mate choice has been studied previously on a few species in clear waters. For instance, in Western Australian seahorse (*Hippocampus subelongatus*) and in Australian seahorse (*Hippocampus whitei*), the matings are size assortative in the wild (Jones et al., 2003; Vincent & Sadler, 1994). In long-snouted seahorses (*Hippocampus guttulatus*), males exhibit a significant preference for larger females during mate choice trials, regardless of familiarity, while females demonstrate a tendency for repeated selection of familiar males over body size preference (Faleiro et al., 2016; Naud et al., 2009).

The dwarf seahorse, *Hippocampus zosterae* Jordan & Gilbert, 1882, is a suitable model species for this study because of their abundance in the wild, including at our study site, Tampa Bay (Masonjones et al., 2019) Their mating system is well documented and their small size makes it feasible to conduct experiments in captivity with manipulation of clear and turbid waters (Masonjones & Lewis, 1996; Rose et al., 2014). The dwarf seahorse belongs to the family Syngnathidae which includes pipefishes, pipehorses, and seadragons. This fish family exhibits unique reproductive strategy of paternal brood care, where males internally fertilize and incubate developing eggs within a specialized brood pouch until live young are birthed (Masonjones & Lewis, 1996). Seahorses are an excellent environmental indicator species because they spend their entire lives in coastal communities, such as coral reefs and seagrass beds, that are threatened by human-induced eutrophication (Shokri et al., 2009). The dwarf seahorse is one of the world's smallest seahorse species, ranging from 16 to 38 mm (Masonjones & Lewis, 1996). Their home range is marked in shallow seagrass beds from the Gulf of Mexico east through the Bahamas, Cuba, and Bermuda (Ginsburg, 1937). Dwarf seahorses have been determined to be socially (Masonjones & Lewis, 1996) and genetically monogamous (Rose et al., 2014), indicating that males receive eggs from one female and remain as a breeding pair. Seahorses exhibit conventional sex roles, meaning that males compete extensively to get access to mates (Vincent, 1994).

Detailed descriptions of behaviors in the dwarf seahorse state that there are four distinct sequential phases of courtship before copulation, which typically occurs in the morning, one or two days prior to copulation day (Masonjones and Lewis, 1996).The behaviors during the consecutive phases serve as potential signals indicating readiness to mate include: phase 1 with reciprocal quivering, phase 2 with male pumping and female pointing, phase 3 with reciprocal pointing, and phase 4 with rising and copulating

(Masonjones & Lewis, 1996). Some studies report that, as in the pot-bellied seahorse (*Hippocampus abdominalis*), dwarf seahorse *(Hippocampus zosterae),* males showcase significantly higher rate of courtship activity than females (Masonjones & Lewis, 1996; Mattle & Wilson, 2009).

In syngnathid fishes, studies showed that population density and sex ratio can affect social interactions including courtship behaviors. For instance, in the case of high fish densities and male biased treatments, male dwarf seahorses tends to show higher levels of activity due to greater levels of aggression and competitive behaviors such as wrestling, snapping, and intruding on courting pairs, leading to potential disruptions in egg transfers and premature brood ejections, ultimately reducing reproductive success (Masonjones & Rose, 2019).

In different model systems, divergent results in terms of mating and reproductive success under turbid environments suggest a multifaceted relationship between eutrophication and its ecological consequences. Given the absence of prior research, this pioneering investigation will be critical in elucidating the ecological consequences of algal turbidity on dwarf seahorses. The specific cues used by dwarf seahorses for mate selection, such as visual and behavioral factors, as well as the potential influence of varying sex ratios on reproductive success under turbid conditions, remain unclear. Hence, this study aims to understand the holistic impacts of turbidity on the mating system of dwarf seahorses across different sex ratios. The central aim of this study is to investigate the effects of algal turbidity on the mating latency, mate choice parameter, courtship behaviors, reproductive success, and gestational duration of dwarf seahorses across varied sex ratios. We initially proposed that turbidity would lead to an increase in courtship behaviors due to reduced visual cues, resulting in enhanced mating and reproductive success (Sundin et al., 2017). We further hypothesized that the murkiness of turbid water would increase mate search time, consequently altering mate choice parameters, as visual recognition of the opposite sex's presence and size would be compromised (Lindqvist et al., 2011; Sundin et al., 2015; Sundin et al., 2010).

METHODS:

Seahorse Collection:

Seahorses were collected from Tampa Bay, Florida, in June-July 2021 and 2022, under special activity licenses SAL-21-2319-SR and SAL-22-2319-SR, respectively. For collecting, push-nets with mesh size of 6 mm with dimensions of 1.5 x 0.91 meter were used. The nets were modified to include zip ties at the bottom of the net, helping to comb through the seagrass beds as reported in Masonjones et al. (2010).

Following collection, seahorses underwent a short-term exposure to freshwater at the University of Tampa's Marine Science Field Station to facilitate the removal of external parasites such as Uronema spp., from the body surface that could possibly impact seahorse behaviors, (MacLean, 2015). Seahorses were transferred into artificial saltwater with a specific gravity of 25-30 ppt, depending upon the salinity of water in the bay during collection. Artificial plants were provided for attachment sites during transport and in holding tanks prior to the study. The seahorses were housed in the aquatic facility at Valdosta State University with the approval of Institutional Animal Care and Use Committee (IACUC) and by the Animal Use Protocol (AUP #00081-2021; Appendix A).

Acclimation:

Seahorses were housed in separate same-sex group tanks in a recirculating system, allowing for visual and chemical cues, for 8-20 days before the experiments as an acclimation period. The light cycle was adjusted to 12L:12D. The room temperature and water temperature were maintained at 26-27°C. Seahorses were fed *ad libitum* twice daily with decapsulated, recently hatched *Artemia*. Water quality was maintained by performing 30% water changes every other day during this acclimatization. The days spent in captivity ranged between 8 to 20 days prior to being used in the experiment.

Culturing algae:

Tisochrysis leutea, a golden-brown, flagellated algae (Bendif et al., 2013) was used to replicate the natural turbid environment. The strain of algae was received from NOAA Laboratories, CT and was cultured in lab. The flowcytometry of the stock (harvested) algae at 10x objective was approximately 8 million cells per milliliter. The algae stocks were provided 24-hour light period to accelerate growth and was diluted to maintain turbidities at 5.67 ± 1.61 NTU in experimental tanks, similar to the turbidity readings reported in Masonjones et al., 2010 for our study location in Tampa Bay during late summers. The readings of turbidity were taken using Oakton T-100 turbidometer, 3 times a day. A standard curve for flowcytometry (cells per million) and SpectroMax (absorbance at 600 nm) in relation to turbidity readings (in NTU) was generated which can further allow the conversion between different turbidity units (Appendix B).

Experimental setup:

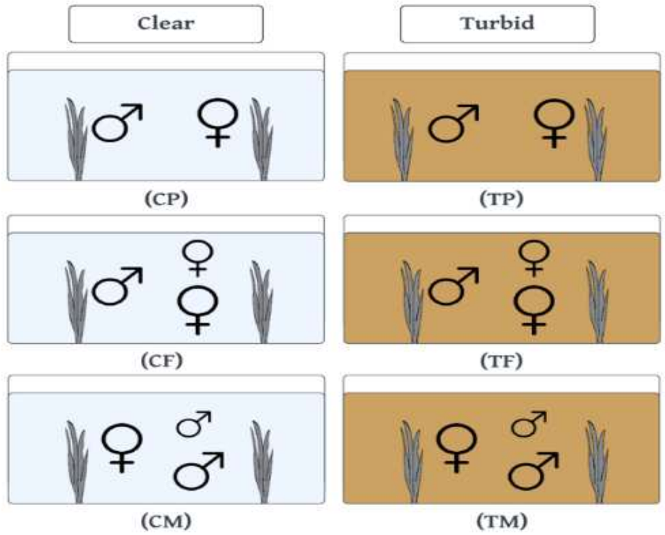


***Figure 1:** Experimental design 3 treatments in clear, C (left) and 3 treatments in turbid, T (right). Top column shows pair treatment (CP and TP), middle column shows female Biased treatment (2F:1M, CF and TF), and last column shows male biased treatment (1F:2M, CM and TM)*

The experiment investigated the impact of algal turbidity on the reproduction of dwarf seahorses in varied sex ratios, resulting in 6 unique treatment combinations namely, paired (1F:1M) in clear (CP) and turbid (TP), Female-biased (2F:1M) in clear (CF) and turbid (TF), and Male-biased (1F: 2M) in clear (CM) and turbid (TM) (Figure 1). The seahorses were kept in a 30 x 21 cm tank with 7 liters of salt water. To avoid visual cues from other tanks, tanks were occluded on all sides but the front.

The water parameters during the experiment were kept similar to the acclimation period. The period of dawn and dusk was imitated by turning on/off the majority of the

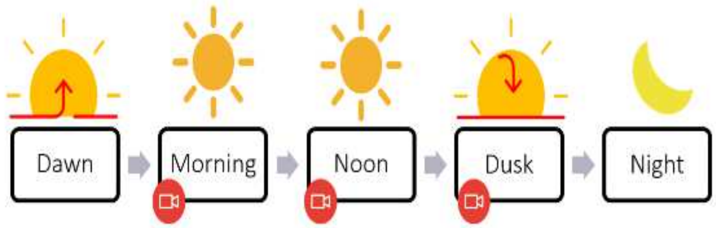


Figure 2: *Graphical representation of experiment timing and video data collection*

room lights to create 30 minutes of a low light environment (Figure 2). Each tank had 2 artificial plants, made by attaching ribbons to the aquarium stones as holdfasts, placed with intervals of 14 cm in the middle of tanks. The rationale behind constructing these plants was to clearly observe all the behaviors without biasing any behaviors that could be missed by bushy plants. This experiment had 8 replications with a total of 128 seahorses used in the mating trials (females, n = 64 and males, n = 64). The first three replications were conducted in summer 2021, and later six replications in summer 2022. The last replicate from the summer of 2021 (replicate #3) was the only replicate conducted in August and hence was not included in analyses to remove any potential impacts of temporal variation, yielding 2 successful replications from 2021 summer. Each fish was used only once in the experiment.

To minimize nutrient variability between clear and turbid tanks, complete water changes were made every day at noon given that brine shrimp (*Artemia*) consumes algae, which can change the seahorse food quality. During this water changes, all the males were checked for eggs by candling the pouch; day of pregnancy was noted if the male had received eggs. The fish were weighed after 24 hours from copulation.

Prior to the onset of each trial, fish were selected using the wet mass measured for all of seahorses. The process included blotting fish dry and weighing in a weigh boat with

1 ml seawater and measured to the nearest milligram. This was followed by photographing seahorses using a digital camera (Olympus Tough TG-6) in a petri dish with a scale, filled with 5 ml seawater, and covered with lid to lay the fish in standard lateral orientation (Figure 2). The fish acclimated in trial tanks for 10 minutes before the experimental filming began. On day 5 of the experiment, each fish was photographed, and fresh weight was measured. On day 10 (from day of copulation), in all treatments, pregnant males were isolated from their tank companions. Females in female-biased treatments were euthanized using MS-222 for performing genetic parentage analysis. After parturition, gestation period along with brood size was noted for each male. Other fish were released back into Tampa Bay in accordance with the SAL protocols. Offspring born in this study were raised for growth and survivorship studies (not reported here), and a small percent of the offspring from the female biased treatments were euthanized using MS-222 for conducting genetic parentage analysis.

The mating latency was determined by counting the number of days from the experiment began until the day of copulation. Mate choice was determined by candling males in male-biased treatments and in female biased treatments, maternal contribution was further confirmed with genetic parentage analysis. Total reproductive efforts were determined by counting the number of fully developed offspring born; 7 out of 47 mated males absorbed the egg mass and did not produce any fully developed offspring, resulting in complete brood reduction.

Body size measurements:

In this experiment, body sizes were approximated by weight. In the sex-biased treatments, each focal fish was given the option of two opposite sex fishes of different sizes, a closely sized matched fish and a distant fish (larger or smaller). The size matched fish had a difference of 0.0176 ± 0.002 g (n = 32), and distant fish had a difference of 0.092 ± 0.0045 g (n=32) compared to the body mass of the focal fish for each treatment. In the paired treatments, the fishes were size matched with a difference of 0.0197 ± 0.0038 g (n=16). The sizes of all fishes in all the corresponding clear and turbid treatments were kept similar to remove any biases (Wilcoxon Rank Sum Test, $n_c = 64$, $n_t = 64$, W = 2039, p-value = 0.9677). Sizes were standardized based on availability of fishes' size range and time from parturition for recently pregnant males. To minimize size disparity between fishes in clear and turbid treatments, body masses were standardized with an absolute difference of 0.0045 ± 0.0005 g (n = 64).

The average body weight of females was slightly bigger than at least one of the males in sex-biased treatments, to parallel the larger average sizes of females collected from the wild. The size of females was also kept in a close range for each replicate to standardize the number of potential hydrated eggs to be transferred to male during experiment, so that that number of offspring born could potentially results from additional competition from other fishes, or due to differential mate choice preference rather than females depositing a varying number of eggs.

Seahorse's body length measurements were digitally quantified using ImageJ program. Snout length, head length, trunk length, tail lengths, standard body length (sum of head, trunk, tail lengths) were measured using the protocols described by Lourie et al.

(1999a, 2003) (Figure 3A). Height was also measured using the live specimen Lourie et al. (1999b) protocol but with a curved approach, starting from tip of the cornet to the tip of tail (shown by green line starting from dotted green line merging into solid line and red line). The body width was measured from the topmost point of the dorsal fin to the widest part of the inferior trunk ridge. Lateral pouch area and crown areas were measured using the area tool as shown in Figure 3B. Morphometric variation between males and females seahorses in the experiment (Appendix C).

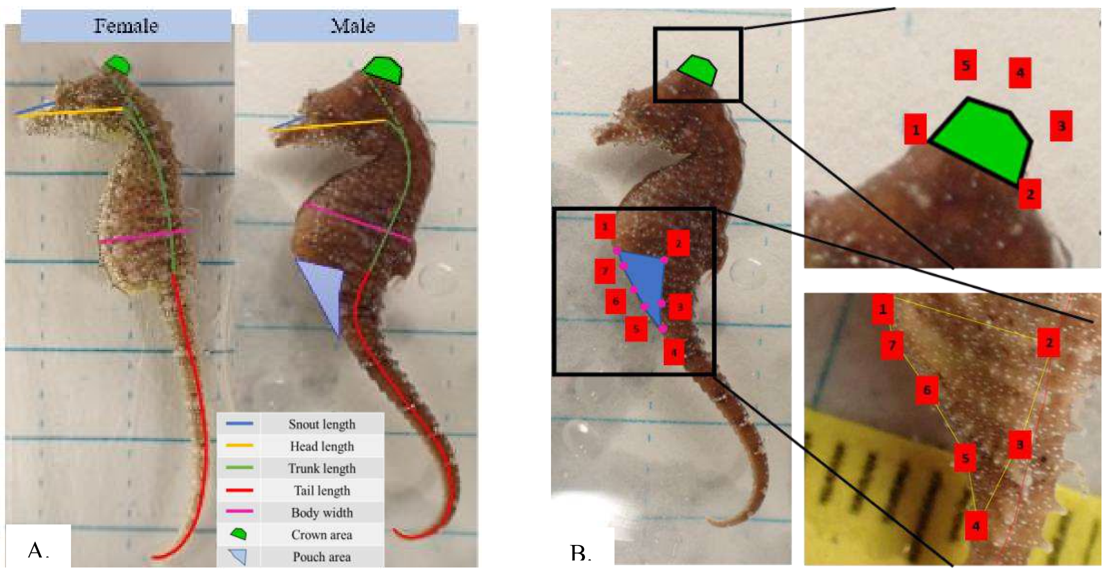


Figure 3: *Seahorse body measurement using ImageJ program.* ***A.*** *Representative images of an adult female (left) and an adult male (right) are shown, with superimposed colored lines and shapes indicating the specific measurements taken.* ***B.*** *The zoomed-in insets provide a clearer visualization of the crown area and pouch area measurements, along with red numbered points that represent landmarks used as specific points of reference within the ImageJ program.*

If copulation did not occur within 5 days, an additional five days were allowed for mating to occur, but no behaviors were recorded during this extension period. If copulation did not happen within these 10 days, then the treatment was terminated (occurred only in one instance, 9TP).

Genetic Parentage Analysis:

To confirm the maternal contribution in female-biased treatments, genetic parentage analysis was conducted for the treatments without brood reduction. We were able to identify the potential mother in the treatments with brood reduction when the copulation was observed and/or by the weight differences among the two females. For DNA extraction of potential mothers, a segment of the tail was used after the fish was euthanized, whereas whole body was used for offspring due to small body sizes resulting in 2 females and 4 babies per treatment. Two microsatellites were used: *Hzos06,* and *Hzos07* and the methods for DNA extraction, amplification of microsatellite followed procedures from Rose et al. (2014). The fragment analysis was conducted at The Cornell Institute of Biotechnology and data was analyzed using Peak Scanner (Thermo Fisher Scientific).

Statistical Method:

All statistical analyses were conducted in Rstudio v. 4.3.1. (R Development Core Team, 2011). We performed model selection of Generalized Linear Mixed Models (GLMM) using lme4 package following the recommendations of Zurr et al. (2009). Each variable's significance was determined by model comparison using ANOVA and backward selection until a minimum suitable model (with lowest AIC value) was reached. Replication number was set as a random effect for each GLMM to account for the variability among the replications unless specified. 9TP was dropped from all analyses because the pair failed to mate in the given experimental time.

1. Latency to mate (in days):

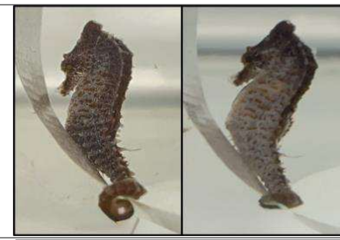
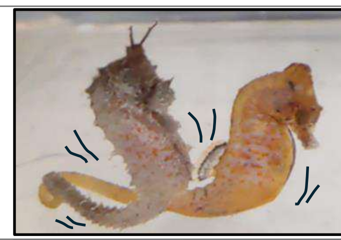
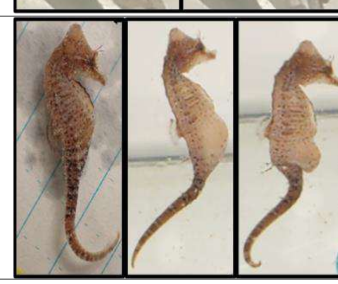
Out of 48 mating trials across all the treatments, 47 pairs lead to a successful mating and 1 pair failed in mating (9TP). Mating latency ranges from 2 to 9 days. In clear treatments, latency averaged 3.42 ± 0.47 days, and in turbid treatments, the latency average was 2.48 ± 0.21 days. To investigate the effects of turbidity, sex ratio, and day in captivity on mating latency, the GLMM model with Poisson distribution and log function was initially constructed allowing 3-way interactions among the variables. However, with backward elimination process, the insignificant terms, including 3-way interaction along with 2 interactions between turbidity-sex ratio and turbidity-day in captivity were removed. The summary of the most significant model is shown in Table 1. To test if there was an effect of body weights of mated fishes (parent fishes) on the mating latency, the GLMM model was conducted with the interaction between weights of male and female. The main effect model (AIC = 179.8) was a better fit model than the interaction model (AIC = 181.8), hence shown in Table 1.

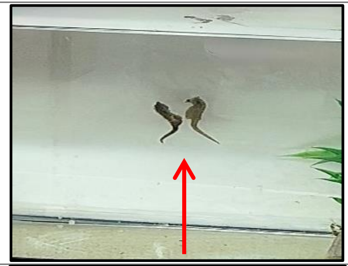
2. Mate choice in sex-biased treatments based on body sizes:

In sex-biased treatments, focal fish were given a choice between two fishes, a size matched and a distant sized fish (larger or smaller) for the opposite sex individuals, resulting in one successfully mated fish and an unsuccessful fish. To determine if mate choice varied due to presence of turbidity was tested by performing Pearson's Chi-squared test with Yates' continuity correction. We were unable to statistically test the mating preference of a focal fish being either size assortatively mated or randomly selected due to low sample sizes across the 4 treatments (Table 2).

3. Mating behaviors:

Figure 3: *Ethogram of the dwarf seahorse mating behaviors*

Mating behavior	Description	Display of behavior
Brightening	Rapid change in body coloration from typical body color to lighter body color over most of the body except the portion of head and dorsal midline remains dark (only recorded for clear treatment)	
Tail holding duration	**Duration:** When male and female fish wrap their tails around eachother's tails and their bodies are oriented in a vertical position	
Initiating Tail holding[f]	**Initiating:** When the fish that initiates wrapping their tail by orienting their tail on the top of the other fish's tail	
Quivering[f]	When fish assume an erect posture, with pectoral fins expanded, and rapidly vibrate their body from side to side	
Pointing[f]	The fish raises its head upward toward the water's surface to form an oblique angle with the main body axis and then lowers it again to a horizontal position.	
Pumping[f] **(male specific)**	Males opening their brood pouch and repeatedly flexing the tail in a motion like that is similar to the behavior displayed during the release of young.	

Rising[f]	Occurs when males and females release their respective holdfasts and rise into the water column facing one another with tails pointing outward while the female's ovipositor and male's pouch start to become aligned.	
Copulatory rise[f]	While rising in the water column, the female genital papilla is placed inside the male brood pouch opening, followed by egg transfer.	
Competition[f] **(male specific)**	Behaviors involving snapping, or same-sex holding tails tightly (Image: Male holding tail snapping at another male)	

[f]Point behavior (frequency of behavior) ⏱**State behavior (duration of behavior)**

Mating behaviors were recorded using GoPro Hero 8 cameras for morning, noon, and dusk for 20 minutes each for 5 days. Courtship behaviors were analyzed using Behavioral Observation Research Interactive Software (BORIS, version 7.9.15) for each subject in our experiment till copulation. The ethogram includes both point events (frequency of event) and state events (duration of event). The point events include non-sex-specific behaviors such as quivering (Q), pointing (Po), rising (R), and male-specific behaviors such as pumping (Pu), and competition (Co). The copulation event was also scored if the event occurred on video. The state events measured by length of time, such as holding tails and brightening, which was scored only for clear treatments due to the visual challenges posed by turbidity that hindered measuring the precise timing of this behavior. We also scored which sex-initiated behavior of holding tails (Figure 4).

Across 47 successful matings, 42 occurred within 5 days of video recordings, and 5 matings occurred after day 5. Videos were analyzed in each tank until the egg transfer. However, in the case of mating after 5 initial days, all their video recordings till day 5 with total observation of 300 minutes per treatment were analyzed. This means that each treatment (tank) was observed for different time points depending on the varying latency time. Hence, to account for time variation, we calculated the rate of behavior with general formula of the number of behaviors observed divided by its respective observation time (in minutes) for all the analysis. In the case of fish mating after 5 initial days, all video recordings until day 5 were analyzed with total observation of 300 minutes per treatment.

The interpretation of mating behaviors was divided into 3 sections: general mating behavior assessments, mated pairs behaviors, and mate choice with respect to mating behaviors.

3.1 General mating behavior assessments:

All the point event behaviors were included for calculating the rate of behaviors (Q, Po, R, Pu, Co) for all individual fish in our study (n = 126). This rate of total point behaviors was tested to quantify the effects of turbidity and sex ratio. The GLMM models were performed using gamma distribution with log function.

We also analyzed the behavioral data separately for each single behavior to quantify the variation due to turbidity, sex ratio, and time of day (3.1a – 3.1c). For this, fish that did not exhibit the given behavior were removed. The behavioral data was sorted that each specific behavior of an individual fish was summed for morning, noon, and dusk and divided by its respective time observed (for quivering: number of fish = 100, number of observations = 160; for pointing: number of fish = 60, number of observations = 69). The GLMM models were performed using gamma distribution with log function.

We also tested the effects of sex, time of day, turbidity and sex ratio treatments on the total number of initiating holding tail behaviors (number of fishes = 77, number of observations = 139). The GLMM model with Poisson distribution with log function was used (3.1d).

Brightening proportion was calculated by time spent by a fish brightening divided by the total time observed for the given time of the day (in minutes) (number of fish = 60, number of observations = 136). The GLMM model was performed to test the effects of sex, sex ratio, and time of the day on the proportion of brightening duration using gamma distribution with log function (3.1e).

3.2 Mated Pairs:

The male and female seahorse that successfully copulated in all our treatments (paired, female-biased and male-biased) are referred to throughout the paper as mated pairs. Our dataset contains 47 mated pairs. This dataset excluded all the unsuccessful fishes from sex-biased treatments. Mated pair's total behavioral rate was determined by adding the rate of total point behaviors exhibited by both the mating male and females (Q, Po, R, Pu, Co) to understand the variation of turbidity and sex ratio treatments. The GLMM model was performed with gamma distribution and log function. The straight effect model was a better fit than the interaction model hence interaction was dropped. In mated pairs, we also analyzed if both sexes perform at a similar rate of behavior to mate. To remove bias arising from sex-specific behaviors of males, such as Pu, and Co, those behaviors were excluded from this analysis. Hence, our rate of non-sex-specific behaviors was tested across sex. The GLMM model was performed using Poisson distribution and log function, with random function consisting of the treatment id (CP, TP, CF, TF, CM, TM) nested with in replicate number.

3.3 Mate choice in sex-biased treatments with respect to mating behaviors:

In sex-biased treatments, mate choice of focal fish was analyzed based upon the behavioral activity of successful and unsuccessful same sex competing fishes. The GLMM model was constructed for the different measurements for rate of behaviors as the response variables with gamma distribution and log function. A nested random effect of treatment id (CF, TF, CM, TM) within trial was included in the GLMM. The predictor variables (fixed effects) were mating success (0 or 1), turbidity, and sex ratio treatments. We tested both the rate of total point behaviors as well as the rate of non-sex-specific behaviors as the response variables. We also quantified the rate of single behaviors, such as quivering, pointing, pumping and competition separately (3.3a-3.3d).

The mate choice was also analyzed in regards with proportion of brightening duration for the successful and unsuccessful fishes among different sex ratios. The GLMM analysis was conducted using gamma distribution and log function with nested random effects of treatment id within trial (3.3e). We also investigated whether the proportion of holding tails (between focal-successful fish, and focal-unsuccessful fish) varied based on mating success, turbidity and sex-biased treatments. The proportion of holding of tail was quantified for focal fish spending time holding tails with successful and unsuccessful fish, divided by the total time observed in minutes. The GLMM model with gamma distribution and log function with nested random effect of treatment id within trial was used (3.3f).

4. Reproductive Success:

Out of the successfully mated 47 pairs, only 40 pairs had reproductive success, while the remaining 7 pairs had brood reduction (1CP, 4CP, 2CF, 8TF, 1CM, 4TM, 9TM). To identify potential cause for brood reduction, GLMM with response variable of reproductive success (0 or 1) along with predictor variables such as turbidity, sex ratio, weight of father, and day in captivity with binomial distribution and logit function was performed. The main effect model (AIC = 56.3) was a better fit than the interaction model (AIC = 59.4). The remainder of the models were initially performed with and without brood reduced treatment being included, but all the models excluding the brood reduction resulted in a better fit with lower AIC values. Hence, all the models were performed excluding brood reduction. To investigate if reproductive output varies across turbidity, sex ratio, and day in captivity, the GLMM model was run with Poisson distribution and log function. The main effect model was a better fit (AIC = 478.8) than the model with the interaction between turbidity and sex ratio (AIC = 481.3).

We also tested the potential effects of parental morphometrics on brood size. We conducted GLMM using 2 parameters: body length and body weights. The dataset only included mated pairs (unsuccessful fishes were excluded). For male body length morphometrics, GLMM model included main effects of standard body length, body width and pouch area. For the female body length morphometrics, the GLMM model included main effects of standard body length, body width, and trunk length. Collinearity was also tested among the morphological variables, and the selected variables have VIF values below 4. The effect of body weight of mated male and female was also tested. In the GLMM model, the main effects model was a statistically better fit (AIC = 463) than the interaction model (weight of mother x weight of father; AIC = 465).

During egg transfer (copulation), females deposit all the eggs from her ovary into the pouch of males (transferred eggs). However, sometimes partial egg transfers are also noted where some eggs do not enter the pouch of the male due to either misalignment or due to the size limitation of pouch area (non-transferred eggs). There were 16 instances in which the non-transferred eggs were documented and counted. To understand the role of turbidity, sex ratio and the relative size of females in sex biased treatments on the number of non-transferred eggs, we tested, 1.) the effects of turbidity and sex ratio treatments on the number of non-transferred eggs; 2.) the effect of relative sizes of female (larger, smaller or size matched) as compared to their respective mated male. The GLMM model with number of non-transferred eggs as a response variable with Poisson distribution was performed.

5. Gestation:

The gestation time in our experiment had very little variation given that for the 40 males with reproductive success, one had a gestation period of 11 days, 14 males for 12 days, 21 males for 13 days, and only 4 males with 14 days. Brood reduced treatments were excluded from all the analysis involving gestation.

RESULTS:

1. Latency to mate (in days):

***Table 1**: Results of GLMMs examining predictors of mating latency in dwarf seahorses. Significant P value is marked with an asterisk*

Predictor	Model	Estimate	Std. Error	z value	P value	
Intercept-Clear, paired	latency ~ turbidity + sex ratio * day captivity	3.07	0.5091	6.03	1.641e-09	*
Turbidity (turbid)		-0.3436	0.1726	-1.991	0.04648	*
Sex biased (female biased)		-1.806	0.7543	-2.395	0.01663	*
Sex biased (male biased)		-1.828	0.7775	-2.351	0.0187	*
Captivity days		-0.1291	0.04044	-3.193	0.001407	*
Sex biased(female biased) * Captivity days		0.1239	0.05643	2.196	0.02811	*
Sex biased(male biased) * Captivity days		0.1169	0.05815	2.011	0.04435	*
Intercept	latency ~ father weight + mother weight	1.469	0.3583	4.099	4.151e-05	*
Father weight		-2.095	1.75	-1.197	0.2314	
Mother weight		-0.2032	2.51	-0.08096	0.9355	

1.1 ***Does turbidity, sex ratio, and the number of days in captivity predict latency?***

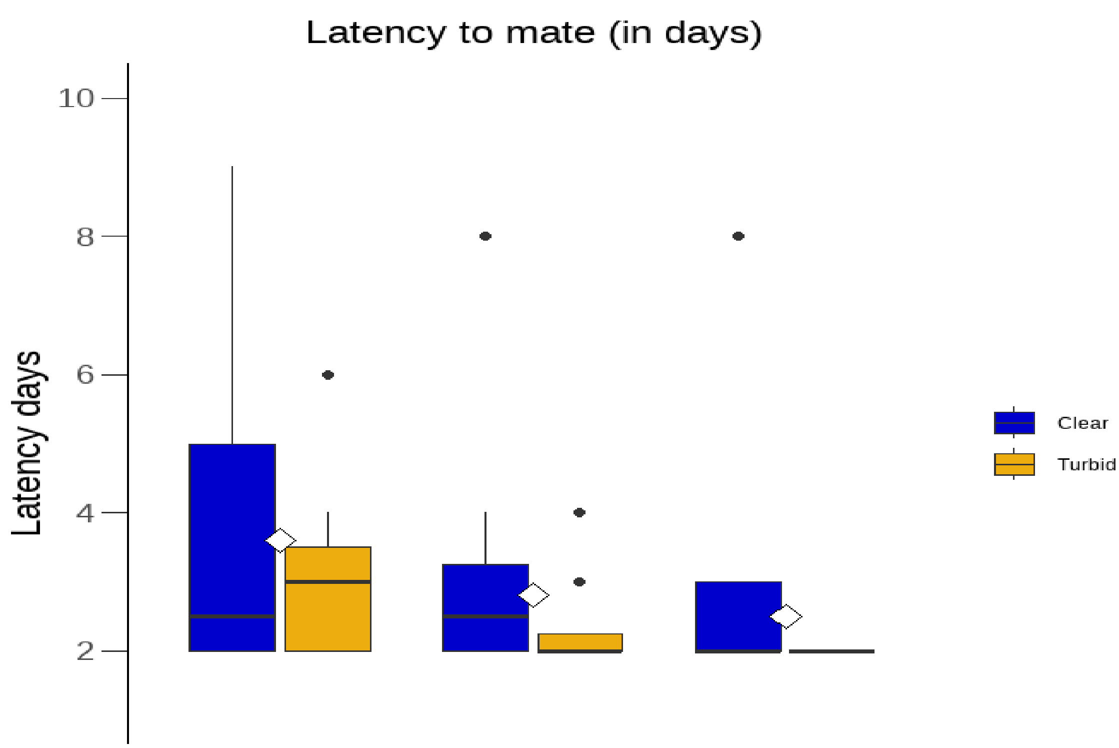


Figure 4: *Latency to mate (in days) across turbidity and sex ratio treatments. Blue and golden boxplots represent clear and turbid treatments respectively. The white diamond shows the mean of the treatment group of sex ratios.*

The statistical results for the most significant model predicting variation in latency to mate are shown in Table 1. The findings indicate that turbidity positively impacts mating latency (GLMM: P = 0.0465; Table 1), resulting in a shorter mating latency in turbid treatments regardless of sex ratio treatments.

The interaction term between sex-biased treatments and captivity was significant such that increasing days spent in captivity, lead to an increase in the mating latency period in sex-biased treatments (GLMM: female biased treatment x captivity days: P = 0.0281; male biased treatment x captivity days: P = 0.0443; Table 1).

1.2 Does latency depend upon the size of parents?

The results for the test to determine if body weight of parent fishes influenced mating latency suggest that there is no impact of the size of either male or female mated fish on the mating latency (Table 1).

2. Mate choice in sex-biased treatments based on body sizes:

2.1 Is mate choice size dependent across varying turbidity and sex ratio treatments?

Table 2: *Mate choice in sex-biased treatments based upon relative size differences*

<table>
<tr><th>Treatment</th><th>Size matched</th><th>Distant</th><th>Total</th><th colspan="2">%Size matched</th><th colspan="2">%Distant</th></tr>
<tr><td>Clear Female Biased</td><td>6</td><td>2</td><td>8</td><td>75</td><td rowspan="2">62.5</td><td>25</td><td rowspan="2">37.5</td></tr>
<tr><td>Clear Male Biased</td><td>4</td><td>4</td><td>8</td><td>50</td><td>50</td></tr>
<tr><td>Turbid Female Biased</td><td>4</td><td>4</td><td>8</td><td>50</td><td rowspan="2">43.75</td><td>50</td><td rowspan="2">56.25</td></tr>
<tr><td>Turbid Male Biased</td><td>3</td><td>5</td><td>8</td><td>37.5</td><td>62.5</td></tr>
</table>

The result suggests that turbidity does not significantly influence whether the fish preferred a size-matched mate or a distant sized mate (Pearson's Chi-squared test with Yates' continuity correction, P > 0.4786). With the low sample size, we did not have statistical power to test if the matings were random, however, the trend suggests that mates are not generally selected based upon body sizes, indicating random mating in terms of body sizes.

3. Mating behaviors:

3.1 General mating behaviors assessments: ***Does rates of behaviors vary among turbidity and sex ratio?***

The intercept of the given model was set for clear, and paired treatments. Turbidity does not impact the rate of total behaviors of the fish (GLMM: -0.030 ± 0.174, P = 0.862). Fishes also behave the same regardless of sex ratio treatment (GLMM female biased: -0.0656 ± 0.2268, P = 0.773; GLMM for male biased: 0.252± 0.2249, P = 0.262).

3.1a: Does turbidity, sex ratio, and time of the day affect the rate of quivering behavior?

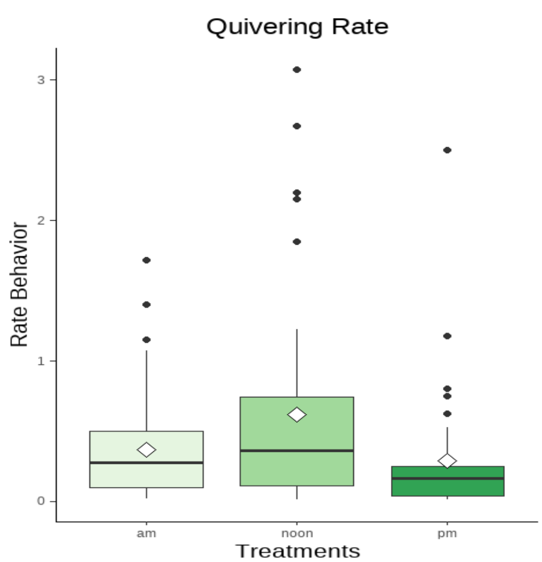


Figure 5: *Quivering rate of all fishes for morning, noon and dusk. White diamond shows the mean of the treatment.*

Quivering rates are similar across all turbid and sex ratio treatments (GLMM for turbidity: 0.021 ± 0.161, P = 0.897; GLMM female biased: 0.293 ± 0.228, P = 0.199; GLMM for male biased: 0.356 ± 0.225, P = 0.113). However, the most significant model shows that quivering rates differ across different times of the day. The rate of quivering is highest at noon as compared to morning and dusk (GLMM for noon: 0.459 ± 0.206, P = 0.026; GLMM for dusk: -0.291 ± 0.2115, P = 0.1686).

3.1b: Does turbidity, sex ratio, and time of the day affect the rate of pointing behavior?

The rate of pointing does not vary among turbidity, time of the day, and sex ratio treatments (GLMM noon time: -0.177 ± 0.231, P = 0.4422; GLMM for dusk: -1.437 ± 0.929, P = 0.122; GLMM for turbidity: -0.291 ± 0.223, P = 0.192; GLMM for female biased: -0.316 ± 0.281, P = 0.26; GLMM for male biased: -0.255 ± 0.294, P = 0.3862).

3.1c: Does brightening duration vary across time of day and sex ratio?

The proportion of brightening also did not vary across different sex ratios (GLMM female biased: 0.231 ± 0.174, P = 0.185; GLMM male biased: 0.1576 ± 0.1719, P = 0.359). The most significant model suggests that the time of the day significantly impacts the brightening display of the fishes. Brightening occurs the most during the morning, as compared to noon and dusk (GLMM for noon: -0.599 ± 0.165, P = 0.0003; GLMM for dusk: -0.807 ± 0.170, P = 2.11 x 10^{-6}).

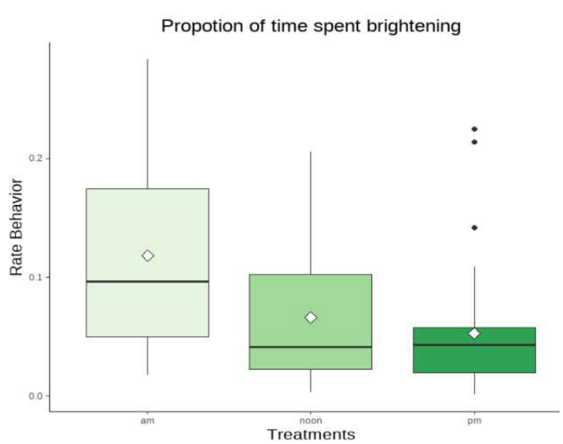


Figure 6: *Proportion of brightening duration of all fishes for morning, noon and dusk. White diamond shows the mean of the*

3.1d: Does total number of tail holding behaviors vary among sex, time of day, and turbidity, and sex ratio treatments?

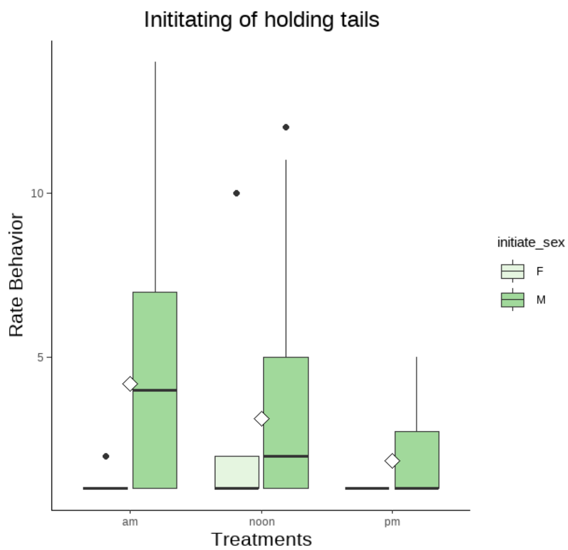


Figure 7: *Initiating holding tails by sex and time of day. White diamond shows the mean of the treatment.*

The results suggest that turbidity and sex ratio do not impact the number of tail holding behaviors (GLMM: turbidity 0.0218 ± 0.094, P = 0.817; sex ratio- female biased: -0.1599 ± 0.131, P = 0.221, sex ratio- male biased: -0.0091 ± 0.113, P = 0.9355), while sex and time of the day does impact the tail holding behavior. Males initiate the tail holding behaviors more than female (GLMM: 0.917 ± 0.179, P = 2.97 x 10^{-7}). Additionally, tail holding behaviors occurred often during morning as compared to noon and dusk (GLMM for noon: -0.3213 ± 0.101, P = 0.00147; GLMM for dusk: -0.9279 ± 0.1632, P = 1.31 x10^{-8}).

3.2 Mated pairs:

3.2.1 Does rate of behavior for mated pairs vary among turbidity and sex ratio?

Behavioral rate of the mated pair is not significantly different across turbidity and sex ratio treatments (GLMM turbidity: -0.028 ± 0.273, P = 0.9166; GLMM female biased: 0.155 ± 0.341, P = 0.649; GLMM male biased: 0.491 ± 0.338, P = 0.1471; Table 3). This indicates that the rate at which mated pairs behave is similar across turbid and sex ratio treatments.

Table 3: *Raw data for the mated pairs's total rate of behaviors across all the replication*

	Paired		Female biased		Male biased			
Trial	clear	turbid	clear	turbid	clear	turbid	Average	Std dev
1	0.1367	0.0000	0.6667	0.6875	0.5786	1.0750	0.5241	0.3945
2	1.2786	0.3714	0.6167	1.4833	1.3750	1.2250	1.0583	0.4526
4	0.8100	0.5650	0.9500	0.4611	0.8714	0.4500	0.6846	0.2193
5	0.8583	1.0500	0.3000	0.6333	2.1500	1.1500	1.0236	0.6307
6	0.7417	0.8333	1.1750	0.3250	0.5778	0.9667	0.7699	0.2975
7	0.1083	0.3889	0.8167	0.8000	0.2733	1.4167	0.6340	0.4775
8	1.5357	0.9654	0.9429	1.8071	2.5750	1.6286	1.5758	0.6049
9	0.3444	NA	0.2458	0.0500	0.0889	0.5786	0.2615	0.2134

3.2.2 In the mated pairs, does behavioral display rate differ among sexes?

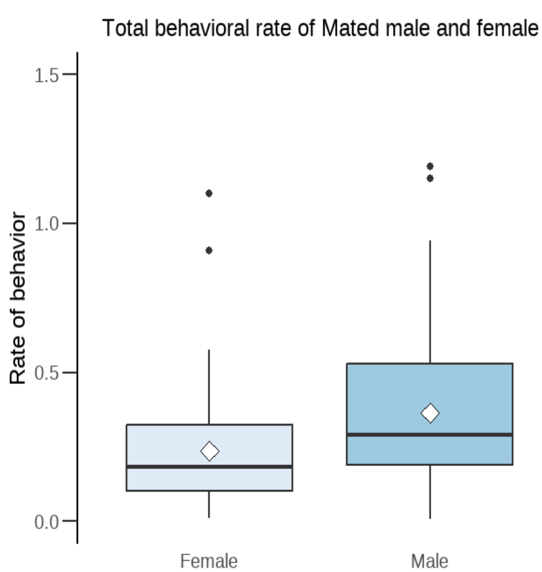


Figure 8: *Rate of behavior in mated pairs by sex. White diamond shows the mean.*

The result indicates that within the mated pairs, males perform significantly more behaviors than their female counterparts, even when male specific behaviors, such as pumping and competition, were excluded (GLMM: 0.4144 ± 0.1392, $P = 0.00291$). This suggest the male's investment in courtship is greater than the females.

3.3 Mate choice in sex-biased treatments with respect to mating behaviors:

Does mating success depend upon total rate of behaviors across the different turbidity and sex ratios treatments?

Regardless of whether the analysis includes sex specific behaviors, the results revealed a significant positive association between mating success and the rate of behaviors (GLMM when excluded sex specific behaviors: 0.873 ± 0.251, $P < 0.0005$; GLMM when including sex specific behaviors: 0.982 ± 0.254, $P = 0.0001$). This reveals that achieving mating success was associated with a higher rate of behaviors on the log scale.

The effect of turbidity was not statistically significant in either of the models (GLMM excluding sex specific behaviors: -0.060 ± 0.239, $P = 0.800$; GLMM including sex specific behaviors: 0.091 ± 0.245, $P = 0.712$), suggesting no significant difference in the rate of behaviors in turbid waters.

However, both the models indicated that male-biased treatments showed a significant positive association with the rate of behaviors (GLMM excluding sex specific behaviors: 0.625 ± 0.233, $P = 0.007$; GLMM including sex specific behaviors: 1.06 ± 0.245, $P = 1.48 \times 10^{-5}$). This means that competing males in male biased treatments group exhibited a significantly higher rate of behaviors compared to two females in the female biased treatments.

Behavioral mate choice

Predictor	Response Variable	Estimate	Std. Error	z value	P value	
Intercept	Rate of total point behaviors (Q,Po,Pu,Co,R)	-2.667	0.3467	-7.692	1.449e-14	*
Mating Success (successfull fish)		0.9818	0.2544	3.859	0.000114	*
Turbidity (turbid)		0.09052	0.2452	0.3692	0.712	
Sex biased (male biased)		1.062	0.2451	4.332	1.479e-05	*
Intercept	Rate of non-sex-specific behaviors (Q,Po,R)	-2.502	0.2976	-8.408	4.174e-17	*
Mating Success (successfull fish)		0.8732	0.2508	3.482	0.0004977	*
Turbidity (turbid)		-0.06039	0.2388	-0.2528	0.8004	
Sex biased (male biased)		0.6248	0.233	2.681	0.007337	*
Intercept	Rate of Quivering	-3.393	0.4229	-8.022	1.04e-15	*
Mating Success (successfull fish)		0.9507	0.3666	2.593	0.009504	*
Turbidity (turbid)		0.1896	0.3144	0.6031	0.5464	
Sex biased (male biased)		1.086	0.3408	3.185	0.001446	*
Intercept	Rate of Pointing	-2.905	0.3923	-7.406	1.305e-13	*
Mating Success (successfull fish)		0.4153	0.3089	1.345	0.1788	
Turbidity (turbid)		-0.1483	0.4653	-0.3186	0.75	
Sex biased (male biased)		0.01828	0.5156	0.03545	0.9717	
Intercept	Rate of Pumping	-3.543	0.5329	-6.648	2.975e-11	*
Mating Success (successfull fish)		1.344	0.2875	4.673	2.97e-06	*
Turbidity (turbid)		0.9247	0.523	1.768	0.07708	
Intercept	Rate of Competition	-2.818	0.6296	-4.475	7.626e-06	*
Mating Success (successfull fish)		-0.1762	0.5558	-0.317	0.7513	
Turbidity (turbid)		-0.7568	0.6064	-1.248	0.212	
(Intercept)	Duration of Brightening	-2.643	0.3189	-8.289	1.142e-16	*
Mating Success (successfull fish)		1.117	0.3085	3.62	0.0002947	*
Sex biased (male biased)		0.0128	0.3293	0.03886	0.969	
Intercept	Duration of Holding Tails	-3.849	0.2816	-13.67	1.6e-42	*
Mating Success (successfull fish)		0.7259	0.2028	3.58	0.0003442	*
Turbidity (turbid)		0.03238	0.2631	0.1231	0.902	
Sex biased (male biased)		0.3753	0.2669	1.406	0.1597	

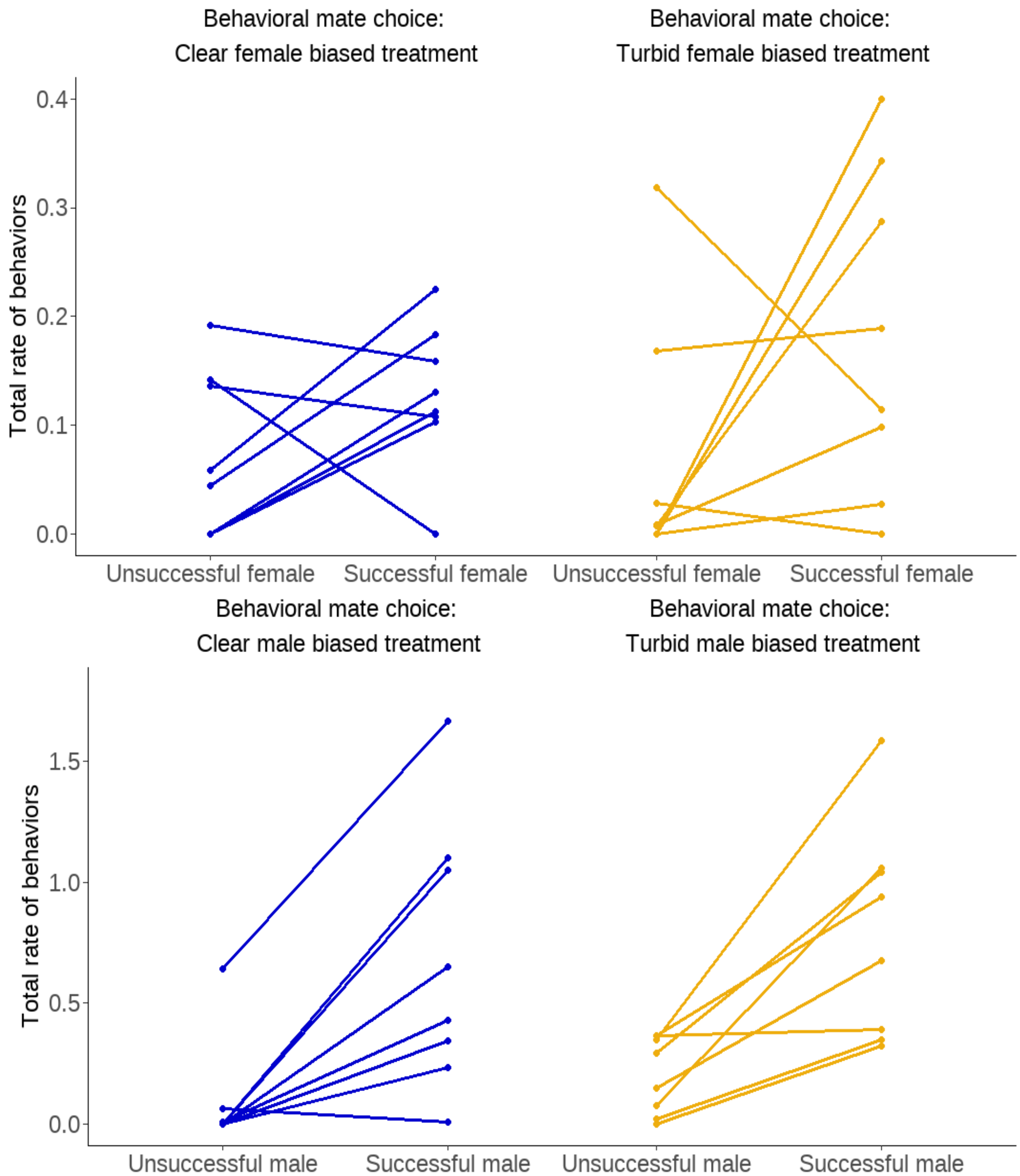


Figure 9: *Mate choice based upon mating behaviors. Blue and golden colors represent clear and turbid treatments, respectively. Top row shows the female biased treatments, showing male mate choice. Bottom row shows the male biased treatments, showing female mating choice.*

3.3a: Does turbidity and sex ratio influence the rate of quivering behavior, and does it differ between successful and unsuccessful fish?

The statistical results revealed a significant positive effect of mating success on the rate of quivering (GLMM: 0.951 ± 0.367, P = 0.0095), indicating that achieving mating success was associated with a significantly higher rate of quivering. There was no effect of turbidity on the rate of quivering (GLMM: 0.1896 ± 0.3144, P = 0.5464), meaning that there was no significant difference in the rate of quivering in turbid and clear waters. However, the male biased treatment showed a significant positive association with the rate of quivering (GLMM: 1.086 ± 0.341, P = 0.0015). This imply that competing males in male biased treatments exhibited a significantly higher rate of quivering compared to the females in female biased treatments.

3.3b: Does turbidity and sex ratio influence the rate of pointing behavior, and does it differ between successful and unsuccessful fish?

The analysis illustrated that the rate of pointing was not statistically different among successful and unsuccessful fishes (GLMM: 0.415 ± 0.309, P = 0.179). Similarly, turbidity and sex ratio also did not vary the rate of pointing behavior (GLMM for turbidity: -0.1487 ± 0.465, P = 0.75; GLMM for sex-biased treatment: 0.0183 ±0.517, P= 0.972).

3.3c: Does turbidity influence the rate of pumping behavior, and does it differ between successful and unsuccessful fish?

The results suggest that the rate of pumping behaviors was statistically different between successful and unsuccessful fishes where the successful male had greater rate of pumping than the unsuccessful male (GLMM: 1.344 ± 0.288, $P = 2.97 \times 10^{-6}$). This indicates that successful mating was associated with a higher rate of pumping behavior. No difference in pumping behaviors were found across the clear and turbid treatments (GLMM: 0.9247 ± 0.523, P = 0.0771).

3.3d: Does turbidity influence the rate of competition behavior, and does it differ between successful and unsuccessful fish?

The analysis revealed that the rate of competition was similar across successful and unsuccessful males (GLMM: -0.1762 ± 0.5558, P = 0.751). Similarly, turbidity did not impact the rate of competition (GLMM: -0.757 ± 0.6064, P = 0.212).

3.3e: Does sex ratio influence the brightening duration, and does it differ between successful and unsuccessful fish?

Brightening duration significantly varied between successful and unsuccessful fish, such that successful fish brightened for greater duration than that of unsuccessful fish (GLMM: 1.117 ± 0.308, P = 0.0003). There was no impact of sex-biased treatments on the proportion of time spent brightening, meaning both male-biased and female-biased treatments showcased brightening at a similar rate (GLMM: 0.013 ± 0.329, P = 0.969).

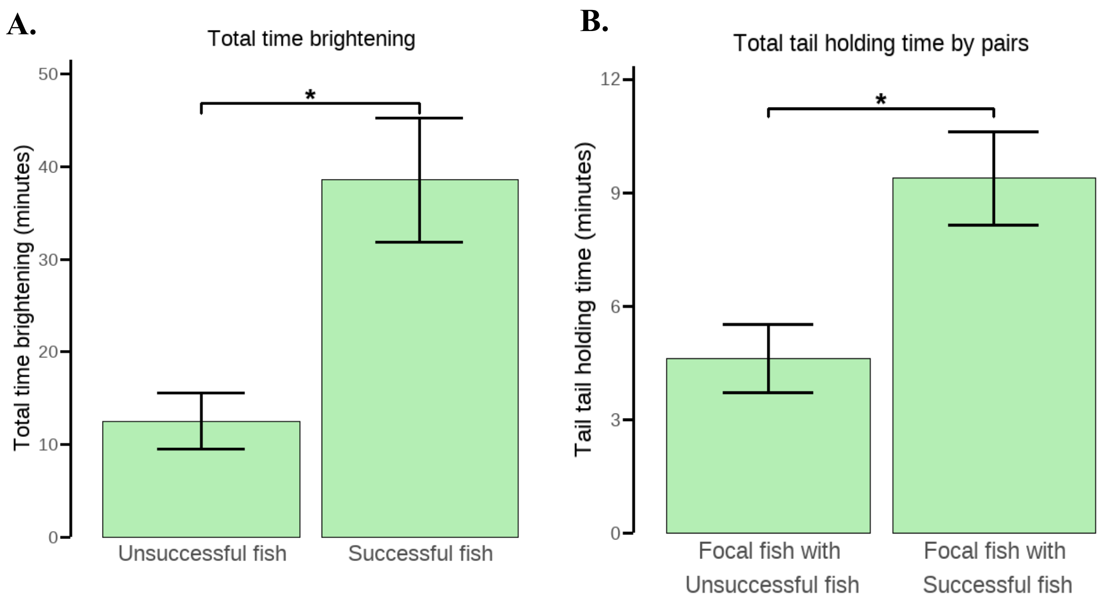


Figure 10: *Behavioral rates vary among successful and unsuccessful fish. A. Brightening proportion between successful and unsuccessful fish. B. Total time spent holding tails between focal fish-successful fish and focal fish-unsuccessful fish.*

3.3f: Does turbidity and sex ratio influence the rate of tail holding behaviors, and does it differ between successful and unsuccessful fish?

The statistics show that successful fish spend a greater amount of time holding tails with the focal fish than that of unsuccessful fish (GLMM: 0.726 ± 0.2028, P = 0.0003). Neither turbidity (GLMM: 0.0324 ± 0.2631, P = 0.902) nor the sex ratio (GLMM: 0.375 ± 0.267, P = 0.160) impacted the duration of brightening.

4. Reproductive success:

Table 4: *Results of GLMMs for reproductive output. Significant P value is marked with an asterisk*

Predictor		Estimate	Std. Error	z value	P value	
Intercept - Clear, paired	Brood reduction	0.2812	1.734	0.1622	0.8712	
Turbidity (turbid)		-0.004519	0.7827	-0.005774	0.9954	
Sex biased (female biased)		0.4937	0.9967	0.4953	0.6204	
Sex biased (male biased)		-0.08937	0.9209	-0.09704	0.9227	
Father weight		4.024	6.418	0.6271	0.5306	
Captivity days		0.04065	0.1027	0.396	0.6921	
Intercept - Clear, paired	Reproductive output	3.513	0.5305	6.622	3.535e-11	*
Turbidity (turbid)		0.03872	0.06102	0.6346	0.5257	
Sex biased (female biased)		0.3436	0.07694	4.466	7.97e-06	*
Sex biased (male biased)		0.3471	0.07716	4.499	6.837e-06	*
Captivity days		-0.03758	0.0366	-1.027	0.3046	
Intercept	Male morphometrics	2.287	0.5083	4.5	6.808e-06	*
Standard body length		0.01949	0.01718	1.135	0.2565	
Body width		-0.01772	0.0822	-0.2155	0.8293	
Pouch area		0.04368	0.01377	3.173	0.001508	*
Intercept	Female morphometrics	0.8714	0.5408	1.611	0.1071	
Standard body length		-0.00246	0.01249	-0.1969	0.8439	
Body width		0.4316	0.07907	5.459	4.777e-08	*
Intercept	Weights	1.797	0.2729	6.583	4.598e-11	*
Father weight		1.31	0.7114	1.841	0.06562	
Mother weight		6.432	1.16	5.547	2.904e-08	*
Intercept - Clear, Paired	Excess eggs dropped-experimental treatments	0.9374	0.3009	3.115	0.001837	*
Turbidity (turbid)		0.7645	0.2421	3.157	0.001593	*
Sex biased (female biased)		0.3668	0.3248	1.129	0.2588	
Sex biased (male biased)		0.1082	0.3496	0.3095	0.757	
Intercept - Relative fem (size matched)	Excess eggs dropped-relative female size	0.3928	0.5821	0.6748	0.4998	
Relative female size (larger)		1.39	0.5983	2.323	0.02016	*
Relative female size (smaller)		1.108	0.6048	1.831	0.06706	

4.1 What factors could lead to brood reduction?

The result shows that none of the variables measured in the study, including turbidity, sex ratio, weight of father, and days in captivity, were identified as a potential cause that predict brood reduction, indicating the occurrence of brood reduction occurred randomly across the treatments (Table 4).

4.2 Do turbidity, sex ratio, or days in captivity influence the number of offspring?

The results show that the sex-biased treatments have significantly greater reproductive output as compared to paired treatment (GLMM: Female biased treatment: $P < 0.0005$, male biased treatments: $P < 0.0005$; Table 4). Brood size is not impacted by the presence of turbidity (GLMM, $P = 0.5257$; Table 4). Days in captivity also do not impact on the size of brood (GLMM: $P = 0.3046$; Table 4).

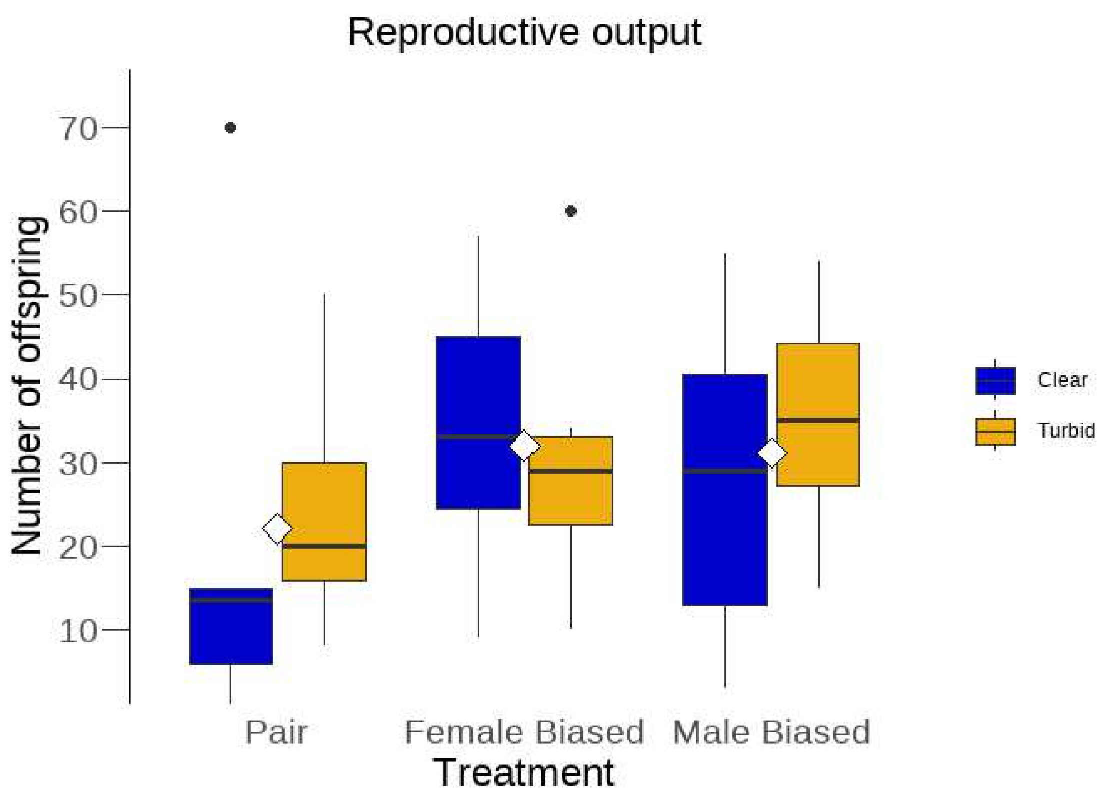


Figure 11: *Reproductive output among paired, female and male biased treatments across clear and turbid treatments. White diamonds represent the means for the groups.*

4.3 Does number of babies correlate with the body morphometrics of parental males and females?

In males, body length analysis suggest that the pouch area is positively correlated with the number of babies born, meaning males with larger pouch area tended to have more babies (GLMM, $P = 0.00151$; Table 4). Other variables such as standard body length (GLMM, P = 0.2565; Table 4) and body width (GLMM, P = 0.8293, Table 4) in males had no impact the reproductive output.

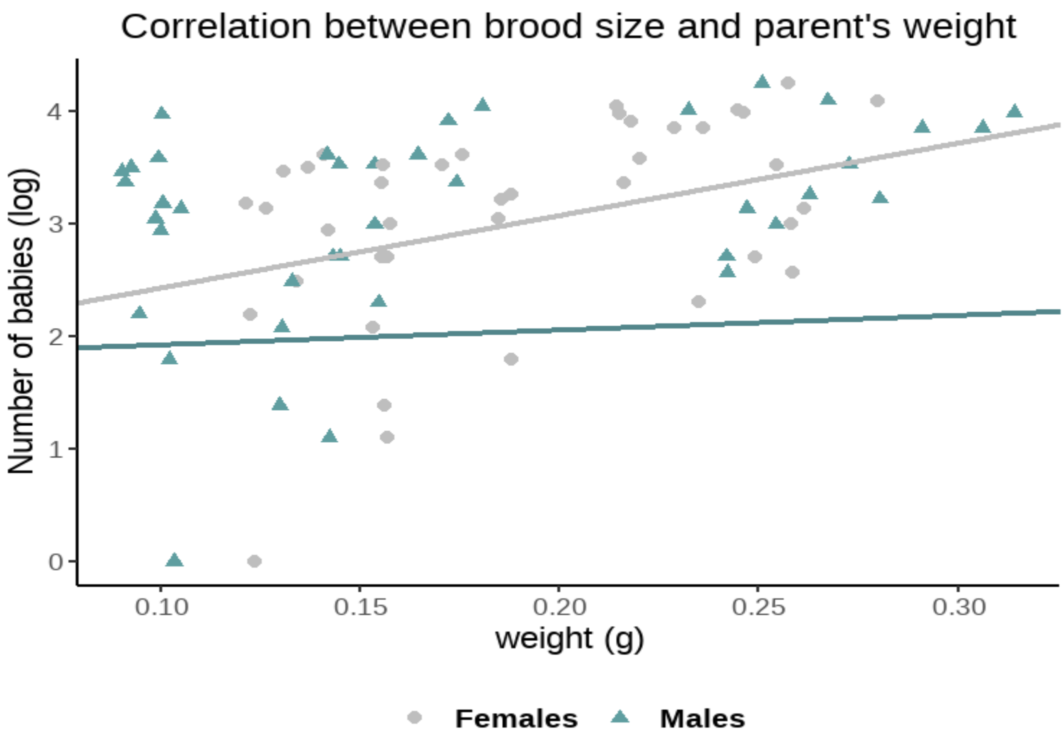


Figure 12: *The correlation between brood size and their parent's weight*

In female, body length analysis suggest that body width is positively correlated with the brood size, meaning females with larger body width have more babies (GLMM, P = 4.73 x 10^{-7}; Table 4). Other variables such as standard body length (GLMM, P = 0.3868; Table 4) and trunk length (GLMM, P = 0.3804; Table 4) did not impact the reproductive output.

For the effect of body mass of mated males and females, the result of the main effect indicates no effect of paternal weight (GLMM, P = 0.066; Table 4), but maternal weight showed a significant positive effect on the number of offspring (GLMM, P = 2.9 x

10^{-8}; Table 4). Hence, this represents that weight of mother better predicts the size of brood, resulting in heavier females producing larger broods.

4.4 *Does turbidity and sex ratio conditions lead to a greater loss of eggs during copulation due to pouch overload?*

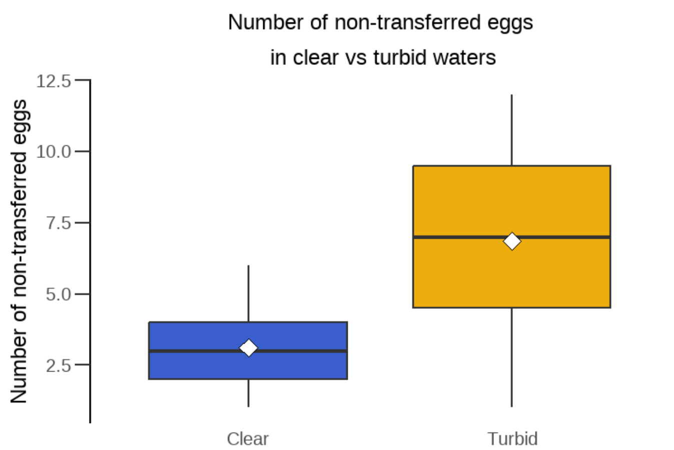


Figure 13: *Number of non-transferred eggs in clear Vs. turbid treatments*

The results suggest that the number of excess eggs dropped from the male's pouch were significantly greater in turbid waters than in clear (GLMM, P = 0.00159; Table 4), although no difference was found among sex ratio treatments.

4.5 Does the relative size of the female (smaller or larger than the male) influence the number of excess eggs that dropped from the male's pouch, in comparison to pairings with size-matched females?

The results show that the number of excess eggs that dropped from relatively larger females are significantly greater than that when males choose size matched females (GLMM, P = 0.0202; Table 4). The number of excess eggs dropped from relatively smaller female is not statistically different from that of size matched females (GLMM, P = 0.0671; Table 4).

5. Gestation:

5.1: How does gestation period vary with water turbidity, sex ratio, and day in captivity?

There was no impact of turbidity, sex ratios and day in captivity on the gestational period of the fishes.

5.2: How does the male behavioral activity of males affect the gestational period?

The behavioral rate of males did not impact the gestational period (GLMM: -0.0003 ± 0.1065, P = 0.998). We also tested the effect of mated pair's behavioral rate on gestation; however, no significance was found (GLMM: 0.00125 ± 0.08, P = 0.987).

5.3: Does gestational period depend upon the reproductive output?

No correlation between the gestational period and the reproductive output was detected (GLMM: 0.00315 ± 0.0026, P = 0.904).

DISCUSSION:

This study provides the first evidence of the impacts of algal turbidity on the seahorse. The influence of turbidity varied in its magnitude across the examined variables. The turbid environment significantly shortened the mating latency period and increased the number of non-transferred eggs. The introduction of turbidity did not alter the seahorses' reproductive output, however given the assumption of the increased amount of non-transferred eggs would have likely enhanced the reproductive output in turbid waters as previously published in Candolin et al. (2016), where hatching success was greater in turbid water. Mate choice in turbid waters is consistent with the clear waters which is based upon the courtship behavioral rate of fish, rather than body size preference (Camargo-dos-Santos et al., 2021). Turbidity also did not alter any behavioral rates of fishes unlike reduced behavioral rate in male guppies and threespine sticklebacks in turbid waters (Camargo-dos-Santos et al., 2021; Candolin et al., 2016).

Latency to mate was significantly shorter in the turbid waters regardless of the sex ratio of fishes, unlike in threespine sticklebacks and broad-nose pipefish where latency to mate is greater due to turbidity (Candolin et al., 2016; Lindqvist et al., 2011). The contradiction with the threespine stickleback is due to reduced courtship behaviors in turbidity, whereas in broad-nose pipefish is likely due to strong sexually selected visual cues for ornamentation in pipefish. Studies on *H. abdominalis*, *H. barouri, H. subelongatus*, and *H. taeniopterus* (but not *H. kuda*) indicate the presence of a *fovea centralis*, which controls visual acuity (Easter, 1992; Lee & O'Brien, 2011; Mosk et al., 2007). However, seahorse visual resolution varies by habitat; for instance, tropical *H. taeniopterus* can detect much smaller food targets than temperate *H. abdominalis*, which are highly adapted to turbid waters (Lee & O'Brien, 2011). Given that in our species, peak breeding season aligns with high turbidity, they are likely to have low resolution vision. This hindered vision may cause 1.) misalignments of gonads between copulating pairs leading to a greater number of non-transferred eggs in the turbid environment than that of clear waters; 2.) a shift towards more efficient non-visual mate detection signals such as tactile responses with holding the tails, and quivering.

In syngnathids, egg production in females and gestational period of males are synchronized. Given that all the male seahorses were caught pregnant from the wild, we allowed time for males to give birth, and females to regenerate eggs in case if she mated recently. For our experiment, fishes also were chosen based on availability of six size matched males and females considering male's parturition period within 20 days. This allowed us to release back majority of fish to the wild within 30 days of capture according to the SAL protocol. Hence the day in captivity ranged from 8 to 20 days. This The effect of captivity on mating latency varies significantly across the fish in the different sex ratio treatments. The two fish in pair setting mate sooner as a result of the increased time spent in captivity, as compared to in sex-biased treatments where there was a longer time prior to copulation as the days increased since the fish were captured from the wild. A possible explanation for the increased latency in sex biased treatments with increase in time in captivity could be the increased number of fishes, which introduced competition, and more time spent investigating the reproductive receptivity of the two potential partners to find an optimal mate to copulate.

Mate choice did not seem to be dictated based upon body sizes in dwarf seahorses as suggested in different species of seahorses such as in *H. abdominalis*, *H. guttulatus*

(Naud et al., 2009; Mattle & Wilson, 2009). This lack of preference may be likely due to the limited overall size variation within the experimental population, as fishes representing sizes from both of the extremes were avoided. This could have potentially prevented salient visual cues for size-based mate choice. Hence, we tested the courtship behavioral rates as a potential parameter of mate choice.

Courtship behavioral rates can be linked to showcase an individual's genetic or parental qualities to their mate while courting and may be an honest indicator of fertilization ability and body conditioning (Moller & Jennions, 2001; Weir & Grant, 2010). When all point event behaviors observed for all of the fishes in the experiment were totaled, turbidity and sex ratio did not impact the total rate of behavior or specific behaviors such as rate of quivering, pointing, and initiating tail holding behaviors. The sex ratio also did not impact the brightening duration of the fishes.

However, when analyzing mate choice based upon behavioral rates in sex-biased treatments, mate choice of both male and female focal fish significantly based upon greater courtship behavioral rates as suggested previously in gulf pipefish by Partridge et al. (2013); threespined sticklebacks by Candolin et al. (2016); in Japanese medaka by Weir & Grant (2010). Along with that, males in the male biased treatment courted at a significantly higher rate than females in female biased treatments consistent with the study of Masonjones & Rose in 2019. This held true even when male specific courtship behaviors such as pumping and competition were excluded (both rate of total point behaviors and rate of non-sex-specific behaviors). Pumping behavioral display is linked with male's readiness to mate. When female mate choice was analyzed based solely upon male's pumping rate, our study provided the evidence where successful males displayed pumping behavior at greater rate than their competing male, suggesting greater reproductive receptivity (Masonjones & Rose, 2019). This is likely an indicator of two important aspects about seahorse courtship. Firstly, males court more which can be an honest indication of male's fertilization ability (Weir & Grant, 2010), and secondly, there is evidence for stronger female preferences towards active males, as shown in sticklebacks and in Japanese medaka (Candolin et al., 2016; Weir and Grant, 2010) (Figure 10). Both reasonings provide a support for conventional sex roles in dwarf seahorses (Masonjones & Lewis, 1996).

State behaviors, such as brightening duration and tail holding duration, also followed similar patterns. The successful fish spent significantly greater lengths of time exhibiting their brightening display as compared to their respective unsuccessful fish in each replicate across treatments. Previous seahorse studies associate brightening activity as establishing social interaction (Vincent, 1994). Similarly, focal fish spent greater time holding tails with the successful fish than that of the unsuccessful fish. This suggests greater social and tactile cues or interactions between fishes before mating were more likely to result in successful copulation attempts.

Male seahorse initiates tail holding behavior at a statistically significantly higher rate than females. This aligns with the observations of the *H. whitei* where male initiated holding female tails more than vice-versa (Vincent & Sadler, 1995). The tail holding action, characterized by one fish wrapping its tail around another, was predominantly initiated and sustained by males for significant periods. Our dataset showed that males have significantly longer tails as compared to females as previously mentioned in Foster & Vincent (2004). This long tail has been previously associated with providing advantage in tail-wrestling behaviors in male-male competition, but our data suggests additional benefits for courting female to enhance tactile cues between fishes (Vincent, 1994).

We had 41 competition instances across 10 males to identify possible female preference based upon competition. Our limited data suggests that female did not show mate preference in regard to the competition rate. Interestingly, male-male competition happened only when matings occurred within first 2 days of experiment. This suggests that greater competition rates might indicate both male's readiness to mate, which can lead to shorter mating latencies in male biased treatments. All of the 17 rising instances were performed by 6 pairs (and one single male) and were observed during the noon timeframe, indicating that courting pairs are most likely to copulate during midday. This is likely to optimize copulation attempts in the presence of the most amount of light availability.

Many of the behaviors in addition to copulation were more likely to occur during specific times of the day. For example, the rate of quivering occurred most during the noon time (noon > morn > dusk), compared to brightening and initiating holding tails was seen primarily during the morning hours. These behaviors are involved in the elaborate sunrise greeting rituals, where males and females in side-by-side positions, hold tails, brightens, and engages in reciprocal quivering and circling around their non-sex-specific holdfast (Masonjones & Lewis, 1996; Vincent, 1994; Vincent & Sadler, 1995). These daily repetition of morning greetings, which continue throughout courtship and gestation period until the male gives birth, serves to facilitate reproductive synchronization of males and females to play a role in establishing and maintaining pair bonding (Gronell, 1984; Vincent 1995). In contrast, the rate of pointing behavior did not statistically vary during the time of the day.

Some of the courtship behaviors in our study also occurred at dusk. Previously reported instances of nocturnal activity are also reported in the lined seahorse (*H. erectus*), Tiger tail seahorse (*H.* comes), Pacific seahorse (*H. ingens*), and in pot-bellied seahorse (*H. abdominalis*) (Foster & Vincent, 2004; Martin-Smith & Vincent, 2005; Mason et al., 2023; Perante et al., 2002).

The rate of total behaviors for the mated pair did not vary among the turbid and sex ratio treatments, meaning all the mating pairs perform at similar rates across different treatments. However, among the mated pair, males' behavioral activity was significantly greater than the females even when the male specific behaviors, such as pumping and competition, was not included (Figure 9). This indicates that males must perform courtship at much greater rate to attract a mate, especially assuming that mating is not size dependent in this species. This remains consistent with the conventional sex roles in seahorses, even in the turbid environment (Masonjones & Lewis, 1996; Vincent, 1994).

As reported by Masonjones and Rose, 2019, an increase in density of males can enhance the chances of atypical behaviors. Our study also has noticed one unusual behavior by a female in the clear, male biased treatments in the morning of copulation. The focal female displayed repeated pumping like behavior for 11 times, which appeared as a mimicking male pumping behavior, either to potentially to encourage the male to continue the pumping behavior for receptivity or was hydrating her oocytes for egg transfer in the morning of copulation as suggested in Foster & Vincent (2004). However, future work is needed to further investigate the given two hypothesis.

Brooding in dwarf seahorses is physiologically demanding for males as they provide extensive care during gestating developing embryos by vascularizing brood pouch lining, osmoregulation, gas exchange and waste removal (Masonjones, 2001). This high metabolic demand, if followed by limited food availability is directly linked to reduced growth rate in brooding males, and increases egg failure, leading to brood reduction. In such instances, brooding male absorbs nutrients from the embryo during gestation leading to no reproductive success (Lin et al., 2024; Paczolt & Jones, 2015). In our experiment, there were 7 out of 47 mating instances where brood reduction was observed (1CP, 4CP, 2CF, 8TF, 1CM, 4TM, 9TM). Our results suggest no significant effects of turbidity, sex ratio, male body mass at the experiment's onset, or duration of captivity on the occurrence of brood reduction. Given that all subjects were fed *ad libitum* twice with freshly hatched *Artemia* resource limitation as a primary driver of brood reduction appears unlikely. Furthermore, to avoid any potential pre-existing reproductive health issues, all males were collected pregnant from the wild. Precautions were also taken to minimize disturbance in handling male during and immediately following the egg transfer. These findings suggest that the observed instances of brood reduction occurred randomly within the experimental conditions.

The brood size was not altered in the turbid waters as previously seen in sand gobies and sticklebacks (Candolin et al., 2007; Järvenpää, & Lindström, 2011). However, sex ratio of fishes had an effect on the brood sizes, such that sex-biased treatments had significantly greater number of babies as compared to the paired treatment. As per authors knowledge, there has been no previous finding suggesting sex ratio alteration that increased reproductive rates. However, considering dwarf seahorses are monogamous, and intending to pair bonds for future matings, the presence of an additional seahorses may increase (indirect) competition rates and attempt to optimize reproductive output. Given the complexity of the rate of brood reductions, along with previous mating and life history of fishes, however, imposes challenges to completely detangle relationships between brood size and morphological characteristics of fishes.

Clutch, egg, and offspring size generally has a stronger positive correlation between female body size in ectotherms (Blanckenhorn, 2005). In seahorses, quantifying maternal and paternal contribution is difficult due to their complex reproductive biology. The results also seem to vary between the species of seahorses given a huge variation in body size ranges (Faleiro et al., 2016; Strawn, 1958; Teixeira & Musick, 2001; Vincent & Giles, 2003; Woods, 2005). However, in this study we attempted to quantify the influence of parental morphology on the brood size in dwarf seahorses. Our results suggest that in females, the body weight and body width of females, but not the standard body length, were statistically correlated with the brood size; and in males, pouch area, but not the weight, length and body width, was statistically correlated with the brood size.

Previous studies have shown pouch volume in males to be significantly correlated with brood sizes in *H. abdominalis,* but not in *H. guttulatus* (Faleiro et al., 2016; Woods, 2005). Biologically, pouch area can be a limiting factor given the varying carrying capacity to hold the eggs received from the female (Dzyuba et al., 2006; Faleiro et al., 2016; Mwale et al., 2014). We also suggested that the matings in dwarf seahorses were not based upon body size, hence given the cases where females were larger than males, when the number of hydrated eggs that could not fit in the pouch of male was significantly greater than when the male mated with size matched female. This suggests the carrying capacity limitation of the pouch area, hence size assortative mating results in optimizing the brood sizes, as suggested by Teixeira and Musick for *H. erectus* (2001).

The brood size is statistically correlated with the body weight and body width of females. As suggested previously for *H. whitei* and *H. erectus*, body weight of females has been shown to be a better predictor of brood size than the total body length (Teixeira & Musick, 2001; Vincent & Giles, 2003). Although previous investigations in *H. zosterae* have reported a correlation between female length and brood size, our analysis did not yield statistically significant evidence of such a relationship, however there was a trend was observed for this pattern (Strawn, 1958). This variation could be likely due to the prior study accounting for dwarf seahorses of all the sizes, whereas our experiment avoided using fishes from either of extreme sizes. Hence, our findings suggest that body weight may be a more effective indicator of the body conditioning of female size as compared to standard body length. We also observed the unsuccessful females in female-biased treatments dropping their entire clutch of eggs after several attempts of matings with the male, which ranged from 20-27 hydrated eggs.

Our results indicate that gestation period was not affected by sex ratio, time spent in captivity, and the presence of turbidity. However, as previous studies have shown, the gestation duration in seahorses depends upon the temperature of their environment, such that the increase in temperature decreases the gestational duration (Foster & Vincent, 2004, Strawn, 1958). As the primary focus of the study was focused on behaviors, mating preferences, and reproductive success under turbid conditions, males were housed in turbidity until 5 days of experiment where water temperatures were maintained at 26-27 ºC, leading to very little variation in the gestation period across the treatments. The males were moved to clear waters after copulation for reminder of the gestation period. Future studies could quantify gestation with males spending entire gestation in turbid waters to test the interaction between turbidity and temperature, along with determining the variation in gestation in wild males across the seasons with varying temperatures and turbidity.

In conclusion, this study reveals a nuanced influence of turbidity and sex ratio on the mating and reproductive system of *H. zosterae*. While water turbidity significantly lowered the latency to mate with increased number of excess eggs dropped, it did not ultimately affect the overall reproductive output. However, sex ratio played a crucial role in reproductive output, with paired treatments yielding fewer offspring compared to sex-biased treatments. Mate choice in this species appears to be driven by behavioral rates rather than driven by body size (suggesting random mate choice based upon body size), hence a greater number of eggs were dropped when females were relatively larger than their mates. Behavioral activity of males was significantly greater than the females. Finally, maternal body weight and width, indicative of potential fecundity, and paternal pouch area, suggesting its capacity influences brood size.

Overall, turbidity did not affect the mate choice parameters, courtship behavioral rate, reproductive outputs and gestational period. This is likely due to the seasonal turbidity recorded year around at the study site in Tampa Bay, Florida, which peaks during late summer and drops at the drops significantly during winter times (Masonjones et al., 2010; Rose et al., 2019). We have investigated the effects of turbidity during peak turbidity and breeding season; however, matings are recorded year around in this species at much lower levels of turbidity. Given the peak breeding season in this species aligns with high turbidity ranges, our results indicate that dwarf seahorses are likely acclimated to the turbid environments. Hence, this pioneer study validates all the previous studies in dwarf seahorses conducted in pristine clear water. The findings of this study will allow us to predict potential changes in the mating system and reproductive success of seahorses under threat from higher fluctuation of human induced eutrophic conditions.

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APPENDIX A:

Institutional Animal Care and Use Committee (IACUC) Approval*

VALDOSTA STATE UNIVERSITY

Institutional Animal Care and Use Committee (IACUC)

Animal Use Protocol Approval

June 17, 2021

Dr. Emily Rose
Department of Biology
Valdosta State University

Dear Dr. Rose;

Animal Use Protocol (AUP) *"The effects of water turbidity on seahorse mating behaviors and reproduction."* (AUP-00081-2021) has been approved by the Institutional Animal Care and Use Committee (IACUC). This approval is from 06.17.2021 – 06.17.2024. Each year, an animal report must be submitted to the IACUC to keep your protocol active. You will be contacted by the Office of Sponsored Programs and Research Administration approximately one month before the annual report is due.

Please remember that you must obtain IACUC approval before amending, or altering the scope, or procedures of the protocol. You are also required to report to attending Veterinarian, the IACUC Chair, and the IACUC Administrator any unanticipated problems with the animals that become apparent during the course, or as a result of the research, or teaching activity.

Should you have questions concerning your approved research, please contact Tina Wright, Compliance Specialist, 229.253.2947, email tmwright@valdosta.edu, or IACUC Alias @ iacuc@valdosta.edu.

Sincerely,

Elizabeth W. Olphie

Elizabeth "Ann" Olphie
IACUC Administrator

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*The approval is based on a group project for research initiated in 2021 via AUP-0081-2021

APPENDIX B:

Standard curve comparing turbidity (NTU) and flowcytometry readings for algal cells.

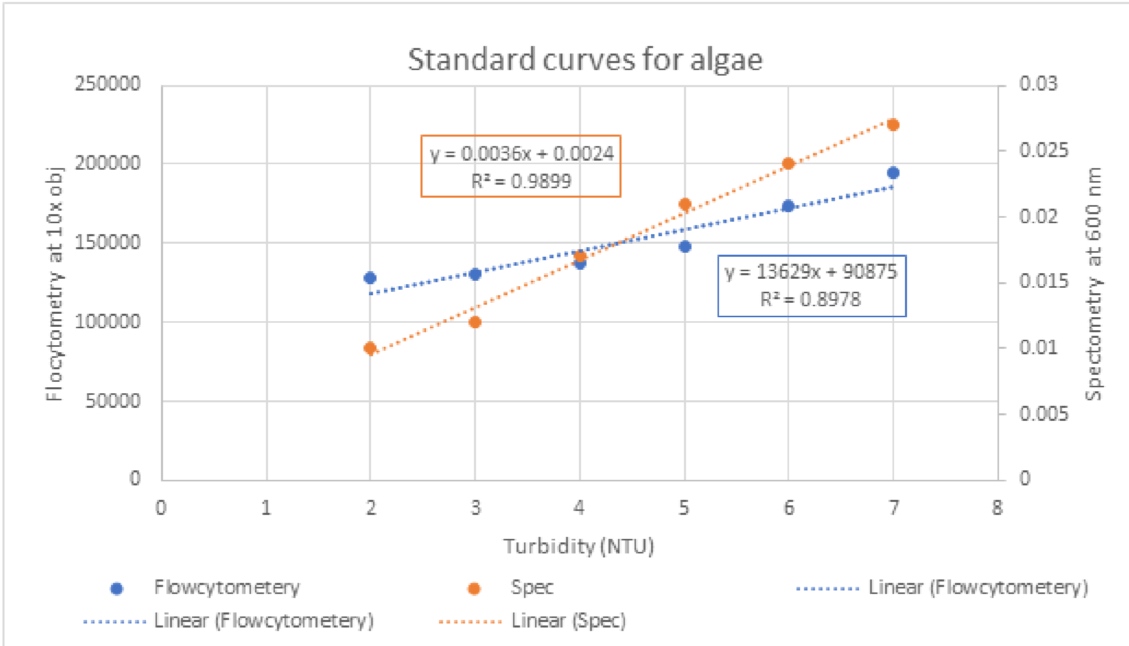

Figure 1. 1 Standard curve comparing turbidity values (NTU) using turbidometer, Flowcytometry readings with number of algal cells at 10x objective lens, and Spectrometry readings with absorbance at 600 nm wavelength

APPENDIX C:

Morphometric variation between males and females seahorses in the experiment

Variable	Sex	n	Mean	Median	Std error	p-value	test
Weight	F	64	0.1876	0.181	0.0061	0.09298	Wilcoxon rank sum test with continuity correction
	M	64	0.1741	0.154	0.0088		
Standard Body Length	F	64	40.0096	39.6905	0.4456	**0.0131***	Welch two sample t.test
	M	64	38.2071	38.719	0.5601		
Height	F	64	36.5412	36.7255	0.4034	**0.1034***	Welch two sample t.test
	M	64	35.4706	35.96	0.5126		
Crown Area	F	64	1.5903	1.5555	0.0430	**0.01101***	Wilcoxon rank sum test with continuity correction
	M	64	1.7725	1.699	0.0551		
Snout Length	F	64	2.6213	2.5705	0.0407	**3.596e-07***	Two Sample t-test
	M	64	2.3085	2.255	0.0416		
Head Length	F	64	6.8203	6.763	0.0782	**6.524e-10***	Two Sample t-test
	M	64	5.9908	5.8805	0.0962		
Trunk Length	F	64	11.9327	11.793	0.1584	**2.2e-16***	Two Sample t-test
	M	64	9.7924	9.857	0.1607		
Tail Length	F	64	21.2566	20.9145	0.2506	**0.02573***	Wilcoxon rank sum test with continuity correction
	M	64	22.4238	22.0505	0.3662		
Body width	F	64	5.6662	5.598	0.0859	**0.03975***	Wilcoxon rank sum test with continuity correction
	M	64	5.9509	5.9145	0.0961		
Total Body Cirri	F	64	6.0938	6	0.5633	**7.13e-08***	Wilcoxon rank sum test with continuity correction
	M	64	2.0469	1	0.3351		
Pouch area	M	63	7.9125	7.23	0.4260	NA	NA