Timing matters: female receptivity and mate choice in the zebrafish (*Danio rerio*)

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Abstract

Female choice has been documented in many animal taxa, and how we test it has been refined through years of studies on the topic. However, when designing mate choice experiments some variables, surprisingly, often remain overlooked, including receptivity and reproductive stage. Here, we aimed to assess whether the female reproductive stage influences strength and direction of mate choice in the zebrafish, *Danio rerio*. Females were offered a choice between two males differing in body size. We found that female choice in our experimental setup was significantly repeatable and that females preferred larger males. Nonetheless, the level of choosiness of females was affected by the time since the last spawning. Females spent more time choosing when tested 7 and 10 days after spawning rather than 4 days, indicating a higher receptivity to males from one week after the last spawning. Moreover, females preferred larger males only when tested 7 and 10 days after spawning. Our results suggest that female mate choice should take female receptivity into account, by standardizing time since the last spawning across females. More broadly, this suggests that 7–10 days since the previous spawning is the ideal time interval for zebrafish female receptivity to peak, with implications for facilities and researchers to increase egg production in natural spawning events and manual egg collection.

Significance statement

The role of pre-copulatory female mate preference has long been recognized in sexual selection. Nonetheless, female receptivity often remains overlooked in mate choice experiments especially in external fertilizing species. In the present study, we investigated if the female reproductive stage affects the strength and direction of female mate choice in an external fertilizing fish, the zebrafish, *Danio rerio*. We found that, when tested 7 and 10 days after spawning rather than 4 days, females spent more time choosing, demonstrating an increased receptivity to males from 1 week following the last spawning. Furthermore, only at 7 and 10 days after spawning females exhibited a clear preference for the bigger males. Our study highlights the importance of considering the female receptivity in future studies assessing mate choices in this and other externally fertilizing species, and also for zebrafish facilities to increase egg production in natural spawning events and manual egg collection.

Keywords Reproductive Cycle · Mate choice · Choosiness · Sexual selection

Introduction

Female preference at pre-copulatory stage has been long studied for its role in sexual selection and the evolution of male mating strategy and sexually selected traits (Rosenthal and Ryan 2022). Assessing female preference in different species is therefore important to study evolutionary processes associated with sexual selection dynamics, and some sort of female choice has now been reported in many taxa (Rosenthal 2017). Much attention has been paid to how accurately female pre-copulatory choice is assessed (for a recent review see Dougherty 2020). It is...
important for reproducibility, accuracy, and interpretation of the results that females are used within their receptive period and at the same stage of the breeding cycle. However, one general issue in female mate choice experiments is how to obtain females with these characteristics, to control the stage of their reproductive cycle, and to ensure the female is responsive to male courtship and willing to exert mate choice. For example, in the Tungara frogs (Physalaemus pustulosus) female choosiness and the criteria used for mate choice change with the stage of the reproductive cycle (Lynch et al. 2006), as well as in the fiddler crab (Uca mjoebergi, Reaney and Backwell 2007). Also, in sailfin mollies (Poecilia latipinna), association times with males vary with female receptivity and only receptive females showed a significant preference for certain male phenotypes (larger males, Ptacek and Travis 1997). Similarly, only receptive females of the African cichlid fish Burton’s mouth-brooder (Astato tilapia burtoni) preferentially associate with territorial males (Clement et al. 2005). Females should therefore be tested at the same stage to ensure not only receptivity, but also preference function and the degree of female choosiness (Jennions and Petrie 1997) may vary with the stage of the reproductive cycle resulting from physiological regulations or adaptive plasticity of female choice.

In many species where females have a temporary fixed reproductive season in the wild, or reproductive season can be recreated and manipulated in the lab (De Vlaming 1972), females could be tested at around the same time of this reproductive window to ensure standardized receptivity. However, in many species, females do not have a temporally fixed reproductive season, so using a fixed time during breeding season is not possible (e.g., Maftei et al. 2016; Deutsch et al. 2017; Todd et al. 2018). Timing of the reproductive cycle can be sometimes assessed using proxies visible from the outside, such as the extension of the abdomen (as it indicates when a female is ready to lay eggs; e.g., Rodewald and Foster 1998; Wesner et al. 2011; Whitney et al. 2012; Quicazan-Rubio et al. 2019). However, the extension of the belly is a trait that is difficult, if not impossible to standardize, even in species when this is clearly visible, due to the variation among females in their body size and the number of eggs each female would produce (Whitney et al. 2012). To overcome these limitations, one possibility is to consider as receptive only those females that spawned with a live male within 24 h after the female choice test (e.g., Mazzi et al. 2003; Frommen and Bakker 2006). Alternatively, females are often used as virgins to ensure standardized receptivity (e.g., Harari et al. 1999; Evans et al. 2004; Sommer-Trembo et al. 2017). However, single-sex rearing is required to obtain virginity, and this may represent a challenge for several reasons, including logistic constraints of space and time, and the necessity to rear females for extended periods in laboratory conditions. Moreover, in iteroparous species virginity can confound the interpretation of results of mate choice because virgin females are also naïve, so this comes with a heightened sexual receptivity associated with a less discriminative approach due to lack of previous experience in, for example, sampling the variety of male phenotypes available (e.g., Houde 1997; Wong et al. 2011; Reuland et al. 2020). In addition, if the female preference is tested by allowing mating, virgin females can be tested only once, which limits the test to a specific age during an individual’s lifetime and prevents repeated tests. Similar limitations are related to the use of sexually deprived females that have been isolated from males for a certain period of time (e.g., McNamara et al. 2004; Cross et al. 2007; Zweerus et al. 2022). To overcome these limitations and to accurately control for receptivity in female mate choice tests, females should thus be tested at the same stage of the reproductive cycle, ideally knowing at what point of her breeding cycle each female is at a specific time. Assuming that the length and duration of the reproductive cycle leading to the subsequent ovulation, and consequently female receptivity, are predictable and can be known with sufficient accuracy, one can rely on the predictable nature of it starting from a point easy to recognize when ovulation is not overt or otherwise difficult to identify. This point could be menstruation that, for example in humans, leads, on average, to ovulation in two weeks (Kerin 1982). Another event that can be used as a landmark of the breeding cycle in many species is the last spawning/laying of eggs. As the natural time interval between two successive spawning/laying is known in many species, the timing from the last spawning/laying is clearly a relatively easy benchmark to control and standardize the stage of the female reproductive cycle (e.g., Lynch et al. 2005; Teuschl and Blankenhorn 2007; Kidd et al. 2013).

In teleost fishes, there is an enormous variety of sexual strategies and mating systems, and female pre-copulatory choice has been well studied in this taxon. This includes fish with external fertilization (the majority of fish species) and with internal fertilization (including the well-studied poeciliid fishes). With a few exceptions, assessing the receptivity of females may be challenging in fishes and indeed this factor has been overlooked in mate choice experiments. It is common, for those fish that can be reared in the laboratory, to use virgin females to ensure maximum receptivity (e.g., guppies, Poecilia reticulata: Evans et al. 2004; least killifish, Heterandria formosa: Asphury and Basolo 2002; mosquitofish, Gambusia affinis: Bisazza and Marin 1991; pygmy halfbeaks, Dermogenys colletti: McNeil et al. 2021; cichlids, Pelvicachromis taeniatus: Baldauf et al. 2013; swordtail, Xiphophorus birchmanni and Xiphophorus malinche: Verzijden and Rosenthal 2011). In livebearing fishes, the reproductive cycle is delimited by parturition events, and
studies have revealed that females are receptive for a short period after giving birth. In guppies, females are receptive for 3–4 days after giving birth and the strength of female choice decreases markedly over this period (Houde 1997). Similarly, sailfin mollys (Poecilia latipinna) are receptive for 2–3 days after producing a brood, after which they become unreceptive to fertilization until after their next brood (Farr and Travis 1986; Snelson et al. 1986; Sumner et al. 1994). In most of the oviparous fish species, females are most receptive around ovulation and, thus, right before spawning (Stacey 1981). If the average interval between natural spawnings is known, knowing the last spawning can be used to standardize female receptivity during behavioral tests. However, in many studies of female choice in oviparous fishes, female mate choice has been tested without controlling for the breeding cycle, possibly making interpretation of female choice difficult.

The zebrafish, Danio rerio, is among the most studied fish species in the world as a model organism for many fields of biological research including development, neurophysiology, and genetics. Despite the huge interest in this small freshwater fish, little attention has been paid to female precopulatory behavior. Nonetheless, there are direct and indirect indications of the occurrence of female mate choice in this species. Indirect evidence includes the fact that the production and release of eggs from a female is highly variable not only between females but also within females, across spawning events of the same individual (Hisaoaka and Firlit 1962). One explanation relies on female mate choice, as females may allocate different number of eggs (clutch size) in each spawning depending on the male they are spawning with, or, in other words, depending on whether and to what extent they prefer that specific male. Direct evidence indicates that females are indeed choosy in this species, and they prefer larger mates (Pyron 2003; Gumm et al. 2009) but do not prefer males with longer fins (Gumm et al. 2009; Kitevski and Pyron 2003). Skinner and Watt (2007) reported a preference for larger males in terms of egg allocation during spawning, with females releasing fewer eggs to small males compared to when spawning with larger males. Another study showed that female preference is also related to olfactory clues with females preferring the odor of unfamiliar and unrelated males, suggesting pre-copulatory inbreeding avoidance (Gerlach and Lysiak 2006). Lastly, two different studies investigated the female preference for novel male characteristics using the GloFish™, a zebrafish mutant line characterized by a red coloration under the dark longitudinal stripes. Snekser et al. (2006) revealed no female preference for red transgenic males whereas Owen et al. (2012) found that females preferred to associate with these males. However, in these studies, females’ reproductive stage was either not standardized or not specifically considered. Moreover, Snekser et al. (2006) did not control the time of the day the female preference was tested, whereas Owen et al. (2012) did so. Indeed, as zebrafish spawn in the morning (Hutter et al. 2010), testing female preference in other time of the day could confound the results of sexual interest with those of social interest.

Sexually mature zebrafish can spawn two or three times each week in the laboratory throughout the year (Eaton and Farley 1974). In laboratory settings, females can even spawn daily for a brief period if the right conditions are in place (Spence and Smith 2005). Nonetheless, this so-called over spawning reduces the amount and quality of eggs, and a week is usually necessary to replenish resources before the next spawning event (Westerfield 1995; Harper and Lawrence 2016). The best breeding frequency suggested for zebrafish when reared in facilities is every 7–10 days (Niimi and LaHam 1974; Westerfield 1995; Harper and Lawrence 2016), as females lay eggs after 5 days since the last oviposition in natural conditions, and optimal spawning seems to be between 5 and 10 days after the previous laying (Hisaoaka and Firlit 1962). This cycle is likely associated with female receptivity, which in turn is associated with ovulation. Corroborating this idea is the finding that females that just spawned eggs show a weaker choice towards males, when mate choice is tested with artificial models (Turnell et al. 2003). It is thus likely that, at least for some aspects, female mate choice in zebrafish, in terms of choice strength and direction, would depend on the timing of the breeding cycle of the female.

In this work, we test the hypothesis that the timing of the breeding cycle (i.e., the time since the last spawning) influences the strength and direction of mate choice in the zebrafish. We adopted a two-choice association testing paradigm, which consists in a female that could choose between two males without physical contact, to minimize the confounding factor of male-male interactions. Indeed, previous findings demonstrated that aggressive male-male interactions can affect female choice in zebrafish when males and females had physical access to each other (Spence and Smith 2005). Additionally, time spent near a male (association time) is a reliable index of female mating preference in several fish species (e.g., Couldridge and Alexander 2001; Gonçalves and Oliveira 2003; Walling et al. 2010), and in the zebrafish (Owen et al 2012). In our study, females could choose between two males that were confined at each end of the test tank. We selected the two competing males presented to the female based on their body size, with one larger and one smaller male, as previous literature revealed that females prefer bigger males (Pyron 2003; Gumm et al. 2009). Before investigating the effect of the female reproductive stage on mate choice, we validated our experimental setup assessing the repeatability of mate choice. After having ascertained the repeatability of female preference in our experimental setup, we tested a different set of females three times
(4 days, 7 days, and 10 days after spawning) to evaluate the strength and direction of female pre-copulatory choice towards the same two males based on female receptivity. We tested females 4 days after spawning since previous findings revealed that this is a non-optimal time for reproduction and 10 days after since this is the last optimal day for reproducing females (Hisaoka and Firlit 1962). We also tested females 7 days after spawning as an in-between day.

**Methods**

**Fish maintenance**

All fish were adult (8-month-old) and experimentally naïve to the apparatus used. Zebrafish were kept in a 400-L opaque plastic tank with natural vegetation and gravel bottom. A 30-W fluorescent lamp (12-h:12-h light/dark photoperiod) illuminated the tank that was also provided with two biomechanical filters. The temperature of the water was maintained constant at 26 ± 1 °C. The fish were fed with commercial food flakes (Aqua tropical, Padovan®) in the morning and live nauplii of *Artemia salina* in the afternoon.

**Mate choice apparatus**

We used the same apparatus of a recent study in which the role of visual and olfactory cues in social decisions of zebrafish was investigated (Santacà et al. 2021). Specifically, the test apparatus (Fig. 1) consisted of five different glass tanks: a central subject tank (female tank), two visual stimulus tanks (males’ tanks), and two olfactory stimulus tanks (for releasing males’ odor into the female tank). A transparent plastic structure internally shaped the focal fish tank (60 × 40 × 32 cm) into an hourglass. A hole at the bottom of the tank allowed the quick and full draining of the tank after each test. The focal fish tank was not directly illuminated, but the two visual stimuli tanks provided indirect lighting thanks to 30 W LED lamps that ensured a 12:12-h light/dark photoperiod and no shaded areas in the female tank. To record the fish behavior, a video recorder (Canon LEGRIA HFR38) was installed above the tank.

The tanks for the males (60 × 40 × 32 cm; Fig. 1) were divided into a smaller front compartment, which was visible to the focal fish (the female), and a larger back compartment, which was equipped with a biomechanical filtering system, green plants (*Hygrophila corymbose* and *Taxiphyllum barbieri*) and a gray-brownish fine grain gravel bottom. Two transparent sliding panels (8 × 5 cm) separated the two compartments. The background of the visible compartments consisted of a white panel to improve the visibility of the stimulus male. Two days before the beginning of the test, one male was inserted into each visual stimulus tank to familiarize with the new environment. During these days, the stimulus males were fed only in the visible compartment, but they were free to move between the two compartments. The same males also served as donors for the olfactory cues. Through a system of pumps, the water from each visual stimulus tank circulated in the olfactory stimulus tank (28 × 19 × 14 cm) that was placed above the males’ tanks (Fig. 1). The odor of each male was released (rate of 3 ml/min) in the middle of the corresponding short wall of the focal fish tank through a transparent small pipe controlled by a valve. A transparent acrylic sheet (40 × 18 cm) placed along the short wall deflected the flow preventing plumes from forming, slowing odor diffusion, and, thus, preventing any odor mixing during each test. In a preliminary test, we visually confirmed odor diffusion in our experimental setup, using methylene blue (which has a comparable molecular mass to the majority of fish pheromones; Chung-Davidson et al. 2010).

![Fig. 1 Plain view of the apparatus showing the set-up of the experimental apparatus used in both experiments](image-url)
Procedure

The stimulus tanks were set up 30 min before the start of the test. Both visual stimulus males were confined in the front compartment of their tank while the female tank was filled to 30 cm with water. The valves of the two olfactory tanks were opened 5 min before the beginning of the test, and, consequently, the olfactory cues were released in the female tank. At the beginning of the experiment, a female was placed in the middle of the tank and her behavior recorded for 30 min. Female zebrafish could thus choose freely between the two males according to their preferences.

Based on the video recordings, we scored the preference of the females. We recorded the time that each female spent in front of the males to assess the preference towards each male. We did this by dividing the total time (30 min) in 5-min periods, which allows controlling for changes in the preference during the test duration. We virtually divided each choice area of the female tank in two: a smaller area (10 cm) and a larger area (20 cm) in front of each stimulus male, indicating, respectively, a stronger or weaker choice. We used a computer software (“Cyclic Timer”, written in Delphi 5 Borland) to measure the time each female spent in each compartment and also counted the number of crosses between the compartments. To minimize observer bias, blinded methods were used when behavioral data were recorded and analyzed. Moreover, to assess inter-rater reliability, an experimenter analyzed blind one third of the videos.

Experiment 1: Repeatability of female mate choice

Repeatability was tested by assaying female choice in two-choice trials conducted 1 day apart at the exact same time (i.e., 7:30, 8:30, 9:30, or 10:30 a.m.). These times of the day were chosen since mating activities in this species occurs in the early morning (Eaton and Farley 1974; Spence et al. 2006; Lawrence 2007), and mating preference tested as association time has been validated for morning (Owen et al. 2012). In each trial, each female was offered a choice between the same two males which differed in body size (mean ± SD difference among male pairs in length: 0.267 ± 0.142 cm). The side of the stimulus males was randomized across the two trials, to avoid any bias in the preference for a side of the tank. Twelve females and six males were used in total.

Experiment 2: Female preference during receptivity window

Female mating preference was tested three times during the reproductive cycle: 4-7-10 days after an initial spawning (time 0). Following standard procedure for zebrafish breeding, one female and two males were transferred to opposite sides of a breeding tank in the late afternoon after feeding. The day after, shortly after the onset of light, the divider between the female and the males was removed and mating could occur. In this experiment, each female was tested three times at the exact same time 4-7-10 days after spawning in the two-choice stimulus tanks to evaluate how the female preference changes with their receptivity (time from spawning). The same pair of males (mean ± SD difference among male pairs in length: 0.256 ± 0.108 cm) was used for each female during the three tests, with the males’ position changed randomly each time. Twelve females, different from those used in the first experiment, and six males, different from those used in the first experiment and from those used for the breeding (to avoid any bias due to previous social experience), were tested in total.

Statistical analysis

Analyses were performed in R version 4.0.5 (R Core Team 2021). To determine inter-rater reliability, we checked for a correlation between the two scorers using the Spearman’s rank approach and found a high inter-rater reliability ($\rho = 0.943$, $p < 0.001$).

We performed similar analyses for the two experiments using linear mixed-effects models (LMMs, lmer function of the lme4 R package; Bates et al. 2015) to analyze female preference (dependent variable: time spent in each compartment after log transformation). Specifically, in the first experiment, the model was fitted with five fixed effects and female ID nested within the male pair as a random effect to account for the non-independency of the tests of the same female and the same male pair. We fitted the model using the male type (larger or smaller) and the area of choice (10 or 20 cm, stronger or weaker choice, see above) as fixed effects. In addition, we included in the model the day (first or second), the time of the day (7:30, 8:30, 9:30, or 10:30 a.m.), and the 5-min blocks as fixed effects to assess whether the female preference changed across days and within the duration of the test. Post hoc pairwise comparisons were performed with Tukey post hoc tests. We also assessed the variability in the choice among females by testing the random-effect term of the LMM (using the “ranova” function of the lmerTest R package; Kunzetsova et al. 2017). We tested whether female preference (using the proportions of time spent with the larger male calculated from untransformed data of each test for each female) and the number of crosses between the compartments were significantly repeatable across successive experimental days using the rptProportion function of the rptR R package (Stoffel et al. 2017). Parametric bootstrapping (1000 iterations) was used to calculate confidence intervals for repeatability estimates whereas likelihood ratio tests were used to determine the statistical significance of the estimates.
For experiment 2, we performed a LMM fitted with the male type (larger or smaller), the area of choice, the day (first, second and third), the time of the day, and the 5-min blocks as fixed effects. Female ID nested within male pair was fitted as random effect. All pairwise comparisons were performed with Tukey post hoc tests. To evaluate variation across females in their preference, we performed a test of the random-effect term of the LMM.

Means are reported with their standard deviations (mean ± SD).

Results

Experiment 1: Repeatability of female mate choice

Overall, females spent 59.76 ± 10.36% of the test time in front of the two males. The average number of crosses from one male to the other was 91.75 ± 32.62 in 30 min of recordings. Females spent 58.83 ± 12.28% of the choice time in front of the larger male on the first day and 55.60 ± 13.51% of the time on the second day.

Females spent significantly more time in front of the larger male \(F_{1,2853} = 71.416, p < 0.001\) without any difference between closer or distant areas \(F_{1,2853} = 1.686, p = 0.194\); interaction: \(F_{1,2853} = 0.317, p = 0.573\). The same model revealed no significant effect of the day (first or second) \(F_{1,2853} = 2.315, p = 0.128\), of the time of the day \(F_{3,2853} = 1.875, p = 0.132\), and of the 5-min time block \(F_{1,2853} = 0.476, p = 0.490\). Nonetheless, the significant interaction between the 5-min time block and male type \(F_{1,2853} = 6.172, p = 0.013\) indicates that during the time of the test, females increase the time spent in front of the larger male. All the other interactions were not significant (all \(p\)-values > 0.35). The test of the random-effect term revealed that females tested with the same male pair did not statistically differ in their preferences for the larger male \(p = 0.997\). Lastly, our repeatability analyses confirmed that female preference in our experimental set up is significantly repeatable (repeatability estimate \(R = 0.684 \text{[CI = 0.200–0.895]}, p = 0.003\)). Also, the number of crosses was repeatable (repeatability estimate \(R = 0.745 \text{[CI = 0.323–0.917]}, p = 0.001\)).

Experiment 2: Female preference during receptivity window

When tested 4, 7, and 10 days after spawning, the average number of crosses from one compartment to the other was 98.58 ± 21.15, 83.58 ± 28.15, and 87.92 ± 33.35, respectively, in 30 min of recordings. The percentage of time (choice time) the females spent in the sectors in front of the two males was 49.92 ± 9.08%, 59.22 ± 12.58%, and 66.19 ± 12.80% on the 4th, 7th, and 10th day, respectively (Fig. 2a). The percentage of time the female spent in front of the larger male was 51.08 ± 6.68%, 61.54 ± 8.09% and 59.13 ± 4.36%, on the 4th, 7th, and 10th day, respectively (Fig. 2b).

Females spent significantly more time in front of larger male \(F_{1,4237} = 70.729, p < 0.001\) with no difference between the near and distant areas \(F_{1,4237} = 0.812, p = 0.368\). Nonetheless, the significant interaction between male and the areas \(F_{1,4237} = 6.172, p = 0.013\) revealed that females spent significantly more time near the larger male in the closer areas (10 cm) \(p < 0.001\) but not in the distant areas (20 cm) \(p = 0.119\). Female preference changed across days \(F_{2,4237} = 7.372, p < 0.001\) but not across the time of the morning in which the test was conducted \(F_{1,4237} = 0.457, p = 0.712\) and across the 5-min time block during the test \(F_{1,4237} = 1.786, p = 0.181\). Specifically, female preference significantly differed when tested 4 days after spawning compared to 7 and 10 days (Tukey post hoc test, 4 vs 7 days: \(p = 0.006\), 4 vs 10 days: \(p = 0.048\), but did not differ between 7 and 10 days (Tukey post hoc test, \(p = 0.353\)). Additionally, the significant interaction between day and area \(F_{2,4237} = 15.605, p < 0.001\) revealed that, when tested 7 or 10 days after spawning, females spent significantly more time in the two sectors closer to the males suggesting a higher receptivity to males compared to 4 days after spawning (Tukey post hoc test, 4 vs 7 days: \(p = 0.022\), 4 vs 10 days: \(p = 0.003\)).
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Discussion

In this work, we tested whether the reproductive stage affects the strength and direction of female mate choice in an external fertilizing fish, the zebrafish. To do so, we first tested the repeatability of female choice in our apparatus, and then assessed the preference of the female with the same pair of males on different days from the previous spawning (4, 7, or 10 days after spawning).

Our results confirmed the repeatability of female choice in our apparatus, and we also confirmed previous findings that females have a pre-copulatory preference for larger males over smaller males (as also previously found by Pyron 2003; Gumm et al. 2009). Female preference for the larger male remained stable across time and there were no significant differences in the direction of female preference among the females we tested (i.e., females preferred the larger male of the pair in the first experiment).

A consistent female preference is a prerequisite for male trait selection to occur, whereas high repeatability of this preference can be used to estimate the maximal value for the heritability of mate preference (Boake 1989). In other animal groups (Møller 1994; Wagner et al. 1995; Johnsen and Zuk 1996), female pre-mating preferences were shown to be variable showing low to high repeatability. Mate choice repeatability has been investigated in few fish species (e.g., Bakker 1993) and, in some cases, provided contradictory results. For example, Godin and Dugatkin (1995) found high repeatability of female preference in the guppy, whereas both Brooks (1996) and Brooks and Endler (2001) found lower repeatability. No previous repeatability estimates were available for zebrafish.

Our main experiment, testing the effect of the reproductive stage on female mate choice, revealed that the female reproductive stage (time since the last spawning) influences not only the strength but also the direction of mate choice in the zebrafish. At 4 days, females spent less time choosing the males (50% of the test) compared to 7 and 10 days (59% and 66%), indicating higher receptivity after 7 days. Moreover, females tested 4 days after spawning did not significantly prefer the larger male over the smaller one. On the other hand, when the female preference was tested 7 and 10 days after spawning, females not only had a significantly higher interest in the opposite sex but also showed a significant preference for the larger males (as also in the repeatability experiment). There is also the possibility that females tested at 7 or 10 days were more used to the experimental arena, thus affecting the choice; however, this is unlikely as in experiment 1, we found a high repeatability across different days, minimizing the possibility that habituation to the experimental setup explains our differences in mating preference among testing days. Thus, our results strongly suggest that controlling for the reproductive stage of females is important in evaluating female mate choices.

Usually, receptivity and reproductive stage are more controlled in species with internal fertilization but should not be overlooked in species with external fertilization. In this latter case, research has focused on those species in which females directly signal their sexual receptivity. For example, female sticklebacks (Gasterosteus aculeatus) exhibit a transient dark mottled or bar-like pattern over their back and upper flanks that is temporally associated with their receptivity to courting males (Rowland et al. 1991). In the Siamese fighting fish (Betta splendens), the presence of vertical bars along female sides has been used as a signal to ensure their receptivity (Dziewczynski and Walsh 2011), whereas in the two-spotted gobies (Gobiusculus flavescens), females have bright yellow-orange bellies during the breeding season (Amundsen and Forsgren 2001). These nuptial colorations have been proposed to be evolved as a signal of readiness to spawn to facilitate efficient mating and reduce male harassment for those females that are not ready to reproduce (Rowland et al. 1991; McLennan 1995). In those species that do not exhibit such colorations and in which sexes do not differ, including the well-known zebrafish, assessing the receptivity of females is not always easy and often this factor has been not controlled for in studies of mate choice. In addition, single female rearing to obtain virginity is not easy for the zebrafish as this species has a low sexual dimorphism that makes them difficult to distinguish before they are sexually mature. Some studies (Snekser et al. 2006; Gumm et al. 2009) tried to control for receptivity by isolating females after sexual maturation, but females may greatly differ in the age (from 3 to 6 months) at which they reach sexual maturity depending on the environmental conditions (e.g., food availability, rearing densities and water temperature; Spence et al. 2008), therefore adding the confounding factor of different age. For this reason, some authors proposed to use body size rather than age to establish reproductive maturity (Eaton and Farley 1974; Spencer et al. 2008). Also, isolating female zebrafish might pose welfare-related issues and may result in health problems due to egg-bound or in changes in social behavior (Hesse and Thünken 2014; Antunes et al. 2021) that could potentially influence their mating preference. Therefore, in testing female mating preference in zebrafish, the reproductive stage is not usually controlled for but can explain the variability in results obtained in this species, for example, the contradictory results found by Snekser et al.
(2006) and Owen et al. (2012) in whether females prefer red transgenic males over the wildtype ones.

From our findings, it appears clear that female receptivity and the reproductive stage are crucial factors to control for when investigating mate choices. Our results have important implications for future studies on the zebrafish mating system and mate choices. Such studies should take into account the female receptive window (7–10 days post-spawning) and standardize the reproductive stage of individuals. Our results also corroborate previous suggestions on how often to use females for breeding purposes, so that 7–10 days seems an ideal period to maximize egg production and quality in natural spawning and manual egg collection. The extensive use of the zebrafish in research demands a comprehensive understanding of this species’ husbandry to ensure effective reproduction and high-standard maintenance of the populations. Once more, this study and others (see, for example, Spence et al. 2008; Hutter et al. 2015) highlight the importance of considering the ecology to better understand zebrafish reproductive biology and behavior and to consequently improve animals’ welfare and researchers’ needs.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00265-022-03255-w.

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Data availability The data in Excel format is available as Supporting Information.

Declarations

Ethics approval Experiments were conducted in compliance with the Italian national law. The experimental procedures were approved by the Ethical Committee of the University of Padova (protocol n. 100/2019).

Competing interests The authors declare no competing interests.

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References

Amundsen T, Forsgren E (2001) Male mate choice selects for female coloration in a fish. P Natl Acad Sci USA 98:13155–13160

Antunes DF, Teles MC, Zauelling M, Friesen CN, Oliveira RF, Aubin-Horth N, Tuborsky B (2021) Early social deprivation shapes neuronal programming of the social decision-making network in a cooperatively breeding fish. Mol Ecol 30:4118–4132

Aspbury AS, Basolo AL (2002) Repeatable female preferences, mating order and mating success in the poeciliid fish, Heterandria Formosa. Behav Ecol Sociobiol 51:238–244

Bakker TCM (1993) Positive genetic correlation between female preference and preferred male ornament in sticklebacks. Nature 363:255–257

Baldauf SA, Engqvist L, Ottenheim T, Bakker TCM, Thünken T (2013) Sex-specific conditional mating preferences in a cichlid fish: implications for sexual conflict. Behav Ecol Sociobiol 67:1179–1186

Bates D, Maechler M, Bolker BM, Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67:1–48

Bisazza A, Marin G (1991) Male size and female mate choice in the eastern mosquitofish (Gambusia holbrooki: Poeciliidae). Copeia 1991:730–735

Boake CR (1989) Repeatability: its role in evolutionary studies of mating behavior. Evol Ecol 3:173–182

Brooks R (1996) Copying and the repeatability of mate choice. Behav Ecol Sociobiol 39:321–329

Brooks R, Endler JA (2001) Female guppies agree to differ: phenotypic and genetic variation in mate-choice behavior and the consequences for sexual selection. Evolution 55:1644–1655

Couldridge VCK, Alexander GJ (2001) Does the time spent near a male predict female mate choice in a Malawian cichlid? J Fish Biol 59:667–672

Chung-Davidson YW, Huertas M, Li W (2010) A review of research in fish pheromones. In: Breithaupt T, Thiel M (eds) Chemical communication in crustaceans. Springer, New York, pp 467–482

Clement TS, Grens KE, Fernald RD (2005) Female affiliative preference depends on reproductive state in the African cichlid fish, Astatotilapia burtoni. Behav Ecol 16:83–88

Cross FR, Jackson RR, Pollard SD (2007) Male and female mate-choice decisions by Evarcha culicivora, an east African jumping spider. Ethology 113:901–908

De Vlaming VL (1972) Environmental control of teleost reproductive cycles: a brief review. J Fish Biol 4:131–140

Deutsch C, Bilenca D, Agostini G (2017) In search of the horned frog (Ceratophrys ornata) in Argentina: complementing field surveys with citizen science. Herpetol Conserv Biol 12:664–672

Dougherty LR (2020) Designing mate choice experiments. Biol Rev 95:759–781

Dzwierzynski TL, Walsh MM (2011) Audience type and receptivity affect male–female interactions in Siamese fighting fish. Ethology 117:10–18

Eaton RC, Farley RD (1974) Spawning cycle and egg production of zebrafish, Brachydanio rerio, in the laboratory. Copeia 1974:195–204

Evans JP, Bisazza A, Pilastro A (2004) Female mating preferences for colourful males in a population of guppies subject to high predation. J Fish Biol 65:1154–1159

Farr JA, Travis J (1986) Fertility advertisement by 469 female sailfin mollies, Poecilia latipinna (Pisces: Poeciliidae). Copeia 1986:467–472
Frommen JG, Bakker TC (2006) Inbreeding avoidance through non-random mating in sticklebacks. Biol Lett 2:232–235
Gerlach G, Lyssiak N (2006) Kin recognition and inbreeding avoidance in zebrafish, *Danio rerio*, is based on phenotype matching. Anim Behav 71:1371–1377
Godin J-GJ, Dugatkin LA (1995) Variability and repeatability of female mating preference in the guppy. Anim Behav 49:1427–1433
Gonçalves DM, Oliveira RF (2003) Time spent close to a sexual partner as a measure of female mate preference in a sex-role-reversed population of the blenny *Salaria pavo* (Risso) (Pisces: Blenniidae). Acta Ethol 6:1–5
Gumm JM, Snekser JL, Iovine MK (2009) Fin-mutant female zebrafish (*Danio rerio*) exhibit differences in association preferences for male fin length. Behav Process 80:35–38
Harari AR, Handler AM, Landolt PJ (1999) Size-assortative mating, male choice and female choice in the curculionid beetle *Diaprepes abbreviatus*. Anim Behav 58:1191–1200
Harper C, Lawrence C (2016) The laboratory zebrafish. CRC Press, Boca Raton
Hesse S, Thünken T (2014) Growth and social behavior in a cichlid fish are affected by social rearing environment and kinship. Naturwissenschaften 101:273–283
Hisaoka KK, Fritil CF (1962) Ovarian cycle and egg production in the zebrafish, *Brachydanio rerio*. Copeia 1962:788–792
Houde AE (1997) Sex, color, and mate choice in guppies. Princeton University Press, Princeton
Hutter S, Penn DJ, Magee S, Zala SM (2010) Reproductive behavior of wild zebrafish (*Danio rerio*) in large tanks. Behaviour 147:641
Jennions MD, Petrie M (1997) Variation in mate choice and mating preferences: a review of causes and consequences. Biol Rev 72:283–327
Johnsen TS, Zuk M (1996) Repeatability of mate choice in female red jungle fowl. Behav Ecol 7:243–246
Kerin JFP (1982) Ovulation detection in the human. Clin Reprod Fertil 1:27–54
Kidd MR, Dijkstra PD, Alcott C, Lavee D, Ma J, O’Connell LA, Kerin JFP (1982) Ovulation detection in the human. Clin Reprod Fertil 1:27–54
Kofler RJ, Spence R, Gerlach G, Lawrence C, Smith C (2008) The behaviour and kinship of wild zebrafish (*Danio rerio*). Anim Behav 83:811–820
Köhn K, Møller AP (1994) Repeatability of female choice in a monogamous swallow. Anim Behav 47:643–648
Koeneke DA, LaHam QN (1974) Influence of breeding time interval on egg number, mortality, and hatching of the zebrafish *Brachydanio rerio*. Can J Zool 52:515–517
Kowhe MA, Rohrer K, Howard RD (2012) Mate choice for a novel male phenotype in zebrafish, *Danio rerio*. Anim Behav 83:811–820
Kracke RP, Lawrence C (2013) The natural history of model organisms: advancing biology through a deeper understanding of zebrafish ecology and evolution. Elife 4:e05635
McAbee MB, Travis J (1997) Mate choice in the sailfin mollie, *Poecilia latipinna*. Evolution 51:1217–1231
McNeil RM, Devigili A, Kolm N, Fitzpatrick JL (2021) Does brain size affect mate choice? An experimental examination in pygmy halfbeaks. Behav Ecol 32:1103–1113
Möller AP (1994) Repeatability of female choice in a monogamous swallow. Anim Behav 47:643–648
Niimi AJ, LaHam QN (1974) Influence of breeding time interval on egg number, mortality, and hatching of the zebrafish *Brachydanio rerio*. Can J Zool 52:515–517
Owen MA, Rohrer K, Howard RD (2012) Mate choice for a novel male phenotype in zebrafish, *Danio rerio*. Anim Behav 83:811–820
Parichy DM (2015) The natural history of model organisms: advancing biology through a deeper understanding of zebrafish ecology and evolution. Elife 4:e05635
Patterson MB, Travis J (1997) Mate choice in the sailfin mollie, *Poecilia latipinna*. Evolution 51:1217–1231
Paton M (2003) Female preferences and male-male interactions in zebrafish (*Danio rerio*). Can J Zool 81:122–125
Quicazan-Rubio EM, van Leeuwen JL, van Manen K, Fleuren M, Pol-lux BJ, Stamhuis EJ (2019) Coasting in live-bearing fish: the drag penalty of being pregnant. J R Soc Interface 16:20180714
R Core Team (2021) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/. Accessed 1 Nov 2021
Reaney LT, Backwell PR (2007) Temporal constraints and female preference for burrow width in the caddis crab, *Uca mjobergi*. Behav Ecol Sociobiol 61:1515–1521
Reuland C, Cumbert BM, Devigili A, Kahrli AF, Fitzpatrick JL (2020) Contrasting female mate preferences for red coloration in a fish. Curr Zool 66:425–433
Rodewald AD, Foster SA (1998) Effects of gravidity on habitat use and antipredator behaviour in three-spined sticklebacks. J Fish Biol 52:973–984
Rosenthal GG (2017) Mate choice. Princeton University Press, Princeton
Rosenthal GG, Ryan MJ (2022) Sexual selection and the ascent of women: mate choice research since Darwin. Science 375:eabi6308
Rowland WJ, Baube CL, Horan TT (1991) Signalling of sexual receptivity by pigmentation pattern in female sticklebacks. Anim Behav 42:243–249
Santaca M, Dadda M, Bisazza A (2021) The role of visual and olfactory cues in social decisions of guppies and zebrafish. Anim Behav 180:209–217
Schlupp I (2021) Mate choice, female competition, and female ornamentation in sexual selection. Oxford University Press, Oxford
Skinner AM, Watt PJ (2007) Strategic egg allocation in the zebra fish, *Danio rerio*. Behav Ecol 18:905–909
Snelder AD, McRobert SP, Murphy CE, Clotfelter ED (2006) Aggregation behavior in wildtype and transgenic zebrafish. Ethology 112:181–187
Snelder FF Jr, Wetherington JD, Large HL (1986) The relationship between interbrood interval and yolk loading in a generalized poeciliid fish, *Poecilia latipinna*. Copeia 1986:295–304
Sommer-Trembo C, Plath M, Gissmann J, Helfrich C, Bierbach D (2017) Context-dependent female mate choice maintains variation in male sexual activity. R Soc Open Sci 4:170303
Spence R, Fatema MK, Reichard M, Huq KA, Wahab MA, Ahmed ZF, Smith C (2006) The distribution and habitat preferences of the zebrafish in Bangladesh. J Fish Biol 69:1435–1448
Spence R, Górski G, Lawrence C, Smith C (2008) The behaviour and ecology of the zebrafish, *Danio rerio*. Biol Rev 83:13–34
Spence R, Smith C (2005) Male territoriality mediates density and sex ratio effects on oviposition in the zebrafish, *Danio rerio*. Anim Behav 69:1317–1323
Stacey NE (1981) Hormonal regulation of female reproductive behavior in fish. Am Zool 21:305–316
Stoffel MA, Nakagawa S, Schielzeth H (2017) rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. Methods Ecol Evol 8:1639–1644
Sumner IT, Travis J, Johnson CD (1994) Methods of female fertility advertisement and variation among males in responsiveness in the sailfin molly (Poecilia latipinna). Copeia 1994:27–34
Teuschl Y, Blanckenhorn WU (2007) The reluctant fly: what makes Sepsis cynipsea females willing to copulate? Anim Behav 73:85–97
Todd MK, Kavanagh RP, Bell P, Munk SA (2018) Calling behaviour of the Tasmanian masked owl Tyto novaehollandiae castanops. Aust Zool 39:449–463
Turnell ER, Mann KD, Rosenthal GG, Gerlach G (2003) Mate choice in zebrafish (Danio rerio) analyzed with video-stimulus techniques. Biol Bull 205:225–226
Verzijden MN, Rosenthal GG (2011) Effects of sensory modality on learned mate preferences in female swordtails. Anim Behav 82:557–562
Wagner WE, Jr, Murray AM, Cade WH (1995) Phenotypic variation in the mating preferences of female field crickets, Gryllus integer. Anim Behav 49:1269–1281
Walling CA, Royle NJ, Lindström J, Metcalfe NB (2010) Do female association preferences predict the likelihood of reproduction? Behav Ecol Sociobiol 64:541–548
Wesner JS, Billman EJ, Meier A, Belk MC (2011) Morphological convergence during pregnancy among predator and nonpredator populations of the livebearing fish Brachyrhaphis rhabdophora (Teleostei: Poeciliidae). Biol J Linn Soc 104:386–392
Westerfield M (1995) The zebrafish book: a guide for the laboratory use of zebrafish (Brachydanio rerio). University of Oregon Press, Eugene
Whitney NM, Pyle RL, Holland KN, Barcz JT (2012) Movements, reproductive seasonality, and fisheries interactions in the whitetip reef shark (Triaenodon obesus) from community-contributed photographs. Environ Biol Fish 93:121–136
Wong RY, So P, Cummings ME (2011) How female size and male displays influence mate preference in a swordtail. Anim Behav 82:691–697
Zweerus NL, van Wijk M, Smallegange IM, Groot AT (2022) Mating status affects female choice when females are signalers. Ecol Evol 12:e8864

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