



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

Maria Santacà¹ · Alessandro Devigili¹ · Clelia Gasparini¹

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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 exhibit 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

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✉ Maria Santacà
santacamaria@gmail.com

¹ Department of Biology, University of Padova, Viale Giuseppe Colombo 3 - Via Ugo Bassi 58/B, 35131 Padua, Italy

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 (*Astatotilapia 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 because 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; Zweerius et al. 2022). To overcome all 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 Blanckenhorn 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*: Aspbury and Basolo 2002; mosquitofish, *Gambusia affinis*: Bisazza and Marin 1991; pygmy halfbeaks, *Dermogenys collettei*: 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 mollies (*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 (Hisaoka 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 (Hisaoka 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.

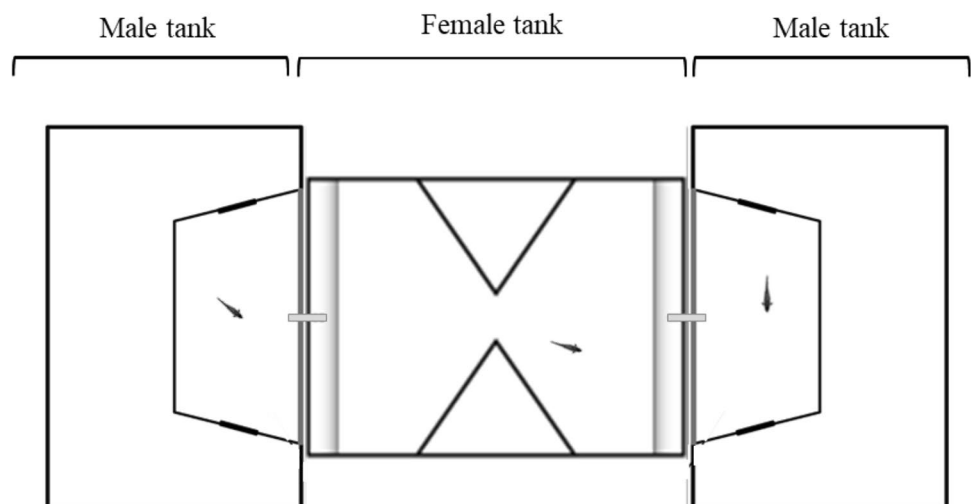
Mate choice apparatus

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 ($60 \times 40 \times 32$ 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 stimulus 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 ($60 \times 40 \times 32$ 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 corymbosa* 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 ($28 \times 19 \times 14$ 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



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 (“Ciclic 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 \pm 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 \pm 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 \pm SD).

Results

Experiment 1: Repeatability of female mate choice

Overall, females spent $59.76 \pm 10.36\%$ 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 $58.83 \pm 12.28\%$ of the choice time in front of the larger male on the first day and $55.60 \pm 13.51\%$ 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 > 0.351). 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 $R = 0.684$

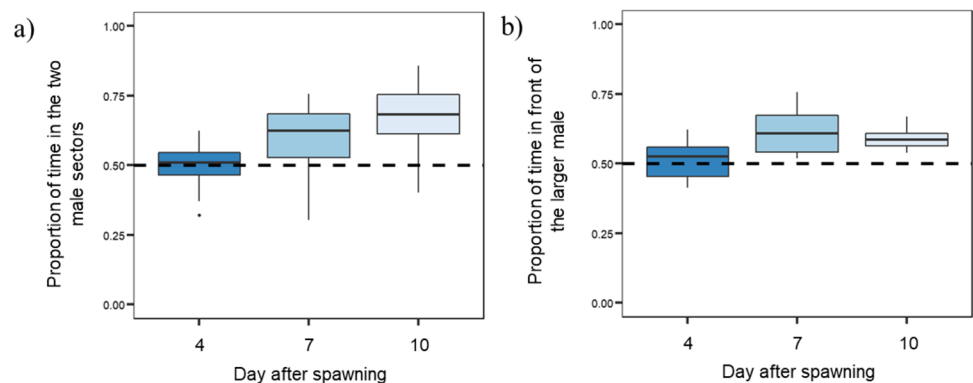
[CI = 0.200–0.895], $p = 0.003$). Also, the number of crosses was repeatable (repeatability estimate $R = 0.745$ [CI = 0.323–0.917], $p = 0.001$).

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 $49.92 \pm 9.08\%$, $59.22 \pm 12.58\%$, and $66.19 \pm 12.80\%$ on the 4th, 7th, and 10th day, respectively (Fig. 2a). The percentage of time the female spent in front of the larger male was $51.08 \pm 6.68\%$, $61.54 \pm 8.09\%$ and $59.13 \pm 4.36\%$, 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 ($F_{3,2853} = 0.457$, $p = 0.712$) 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:

Fig. 2 Results of the experiment 2: **a)** proportion of time (calculated from untransformed data) females spent in the two male sectors; **b)** proportion of time (calculated from untransformed data) females spent in front of the larger male. The box plots report median, lower and upper quartiles; whiskers represent values within 1.5 times the interquartile range and individual points represent outlying values



$p=0.009$). No significant difference emerged between 7 and 10 days after spawning (Tukey post hoc test, $p=0.219$). All the other interactions were not significant (LMM, all p -values > 0.398). The test of the random-effect term of the LMM revealed that females tested with the same male pair did not statistically differ in their preferences ($p=0.977$).

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 (Dziewieczynski 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. 2010; Parichy 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.

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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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