Mate preference in sympatric and allopatric darters (genus *Etheostoma*): comparative evidence for a sex effect in reinforcement

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Abstract

Reinforcement is the strengthening of preference for conspecific mates as a byproduct of selection against hybrids. A hypothesis of reinforcement predicts that populations evolving in sympatry will have greater preferences for conspecific over heterospecific mates as compared to allopatric populations. In animals, this pattern was demonstrated across the genus *Drosophila*, however, most empirical evidence for reinforcement comes from studies of two or only a few species. To infer general patterns requires broader comparative analyses. We therefore compared mate preferences in sympatric and allopatric species of darters (genus Etheostoma). We performed a meta-analysis of 15 studies, encompassing 20 species. As expected, we found stronger preferences for conspecifics over heterospecifics across studies, species, and sexes. We further found the classic signature of reinforcement, with sympatric species showing on average stronger preferences for conspecifics than allopatric species. This was especially true for females, consistent with the idea that mate choice might be more costly for females than males.

Introduction

As evolutionary biology continues to explore the mechanics of speciation, reproductive isolation remains a fundamental question. Reproductive barriers that reduce gene flow among evolutionary lineages can happen at the prezygotic and postzygotic stages. Prezygotic barriers may include environmental (habitat differences), behavioral (mate preference divergence), mechanical (genitalia differences), and gametic incompatibilities. Postzygotic barriers involve selection against hybrids, in the form of lower fitness for hybrid offspring, such as sterility or inviability. The two types of barriers interact in the process of reinforcement when selection against hybrids (postzygotic barriers) favors an increase in prezygotic barriers (Dobzhansky, 1940). This reinforcement of prezygotic isolation usually results in differences in courtship signalling phenotypes (i.e., reproductive character displacement). Its classic signature is a greater divergence of a courtship phenotype between species in areas of sympatry than allopatry (Rundle & Schluter, 1998). For

instance, species-specific courtship signals and a preference for conspecifics should be stronger within sympatric populations than within allopatric populations.

While the prediction of reinforcement is straightforward, the theory has been widely debated (for a review, see (Howard, 1993) as to whether the theoretical assumptions are correct (Mayr, 2013) or whether sufficient evidence exists in nature (see e.g. Paterson, 1978; Butlin, 1987; Rice and Hostert, 1993). When (Liou & Price, 1994) proposed a convincing multilocus genetic model, the theory regained popularity.

Building on this renewed attention, several studies sought experimental evidence to validate the hypothesis. Their findings indicate an increased preference or signal divergence when closely related species overlap, the typical behavioral signature of reinforcement. For example, (Rundle & Schluter, 1998) investigated mate choice in the threespine stickleback (*Gasterosteus aculeatus*) and found that benthic sympatric females preferentially mated with their own type whereas females from allopatric populations showed no preference between males of their own and the other ecotype. In two species of White-eye birds (*Zosterops poliogaster* and *Z. abyssinicus*) that can occur in sympatry, parapatry or allopatry, contact calls were shown to be more distinct between both species when they are sympatric than within allopatric or parapatric populations (Husemann et al., 2014). In the green tree frog (*Hyla cinerea*), females from sympatric populations had stronger preferences for conspecific calls and greater call discrimination abilities than females from allopatric populations (Höbel & Gerhardt, 2003). Those results provide convincing evidence for reinforcement but, being limited to single pairs of species or populations, they do not make it possible either to evaluate whether the process is general or idiosyncratic nor to study the factors that favour the appearance of reinforcement and its magnitude. Comparative studies with multiple species are needed to address these questions.

The work of (Coyne & Orr, 1989, 1997) in *Drosophila* was the first of its kind to bring empirical evidence of the presence of reinforcement at the genus level. They found that prezygotic barriers (mate choice), but not postzygotic barriers (hybrid inviability and hybrid sterility), were twice as strong in sympatric species than in allopatric species. Adding to those seminal results, Yukilevich & Peterson (2019) found that female *Drosophila* had stronger preferences for conspecific males than males did for females in sympatric species, whereas no sex difference in preference was found in allopatric species. At a smaller scale, (Lemmon, 2009) compared mating preferences in several populations of two frog species that occurred both in sympatry and in allopatry. Lemmon found that, compared with allopatric females, sympatric females more strongly preferred conspecific signals and this preference was stronger when the conspecific signal was the sympatric one. Comparative studies in plants also support a pattern of reinforcement, though Hopkins (2013) points out ways in which those studies could be strengthened.

Here, we focused on darters (genus *Etheostoma*) to perform a comparative analysis of reinforcement and to explore the role of sex in that process. Darters are a large clade of North American freshwater fishes characterized by elaborate secondary sexual traits (Page & Burr, 2011). Mate preference and mate choice have been investigated in several pairs of species in darters. Studies find that species demonstrate a varying degree of preference for conspecific over heterospecific mates (Martin & Mendelson, 2013; Williams & Mendelson, 2013; Mendelson et al., 2018) and that mate preference or choice for conspecifics is present in both females (Williams & Mendelson, 2010, 2011; Roberts et al., 2017) and males

(Ciccotto et al., 2013; Zhou et al., 2015; Martin & Mendelson, 2016; Moran et al., 2017; Roberts & Mendelson, 2017; Moran & Fuller, 2018), depending on which heterospecific is presented. Moreover, the presence of hybridization has been documented in a few darter species (Bossu & Near, 2013), making this clade a good study system to examine reinforcement at the genus level.

Two studies of darters have tested explicitly for evidence of reinforcement in one or a small number of species pairs. Moran & Fuller (2018) compared male choice and aggressive behaviors in a small number of closely related species (*Etheostoma caeruleum* and members of the *Ceasia* species complex). They found that both male preference for conspecific females and aggressive behaviors towards conspecific males were stronger in populations that were sympatric with the congener. Roberts & Mendelson (2020) measured the strength of preference for conspecifics in allopatric and sympatric populations of two darter species (*Etheostoma zonale* and *E. barrenense*). They also found a stronger preference for conspecific mates in sympatric populations, but only in females, which contrasts the results of Moran and Fuller (2018), who found evidence of reinforcement only in males.

In this paper, we performed a phylogenetically informed meta-analysis to determine the extent to which increased preference for conspecific mates in sympatry, the classical signature of reinforcement, is present in *Etheostoma* darter fishes, a diverse genus of stream fishes. We hypothesize that differences among species in preference for conspecifics are driven by geographic relationships, predicting that sympatric species will have a stronger preference due to reinforcement. We also compared the strength of preference for conspecifics between males and females. Most sexual selection theory predicts that females will be choosier (Darwin, 1871; Trivers 1972; Andersson, 1994 but see Edward & Chapman, 2011), and Yukilevich & Peterson's (2019) results in *Drosophila* are consistent with that prediction. However, given abundant evidence of male mate choice in darters, and the contrasting results of two studies of reinforcement, the relative importance of male and female mate choice in reinforcement in general remains an open question.

Material and Methods

Inclusion criteria

We conducted our meta-analysis on studies produced by a single lab over the past twelve years (2010-2022). We included 12 published papers (indicated with an asterisk in the reference section) and three unpublished datasets, encompassing 20 species of *Etheostoma* distributed across the phylogeny of the genus (Near et al. 2012), arranged in 13 different pairs of species.

All studies used a dichotomous mate preference paradigm where individuals do not have physical access to one another. The main measure of preference is the time the focal fish spends in an association zone adjacent to either a conspecific or a heterospecific individual of the opposite sex (figure1). Most studies allowed only visual cues, with focal and stimulus fish separated into different tanks. Two studies (O'Rourke & Mendelson 2010 and Barber &

Mendelson, unpublished) used partitions to separate focal and stimuli fish that were not water-tight, thus potentially allowing exchanges of chemical cues.

This conservative choice of experimental design removes aggressive physical interactions among members of the same sex as an explanation for the behavior of the focal fish. It also emphasizes mate preference rather than mate choice, as choice is better measured by allowing physical access to potential mates (as in e.g. Moran et al., 2017, Zhou et al., 2015).

Effect size calculation

All included studies measured the time spent in the association zones. Sample sizes, means and standard deviations were extracted from each paper and when those variables were not available, we contacted corresponding authors to obtain the raw data. The effect size was calculated as a Pearson's correlation coefficient *r* of times spent in conspecific association zones minus heterospecific association zones for each tested species and sex of each study (Equation 1):

$$r = \frac{d}{\sqrt{d^2+\frac{1}{p*(1-p)}}}, \text{(Equation 1)}$$

$$\text{with } d = \frac{\bar{\mu}_{sample_C} - \bar{\mu}_{sample_H}}{\sigma_{pooled}}, \; p = \frac{N_{sample_C}}{N_{sample_C} + N_{sample_H}},$$

$$\text{and } \sigma_{pooled} = \frac{\sigma_{sample_C}{}^2 * (N_{sample_C} - 1) + \sigma_{sample_H}{}^2 * (N_{sample_H} - 1)}{N_{sample_C} + N_{sample_H} - 2}$$

where $\bar{\mu}_{sample}$ and σ_{sample} correspond to the mean and standard deviation of the time spent with conspecifics ($\bar{\mu}_{sample_C}$) and with heterospecifics ($\bar{\mu}_{sample_H}$), N_{sample} corresponds to the sample size of tested individuals, which is identical for both conspecifics and heterospecifics in our case. We did not Z-transform our correlation coefficient as this can affect the accuracy of statistical models (see Janicke et al., 2019 for a similar decision).

Moderators and their rationale

We sought to determine which factors might influence the strength of preference for conspecifics over heterospecifics, as represented by the effect size. We selected three 'natural' factors: geography, genetic distance or relatedness, and sex of the tested individual, and three 'experimental' factors: the size of the association zones, stimulus type, and recording duration times.

Geography: Aligned with the reinforcement hypothesis (Dobzhansky, 1940; Howard, 1993), we predict the geographic relationship to influence the strength of preference for conspecifics, with a stronger preference in sympatric species. For each study, we determined whether species pairs were allopatric or sympatric (Lee et al. 1981; Page 1983; Etnier and Starnes 2001) and included this variable as a moderator. Some species pairs

consist of both allopatric and sympatric populations (i.e., incomplete range overlap); pairs were scored according to the population of origin.

Sex: Although classical sexual selection theory predicts a stronger preference in females, some studies have found the opposite pattern, with a stronger preference for conspecifics in males compared to females (e.g., Mendelson et al., 2018; Moran & Fuller, 2018). Our dataset includes as many male-focal individuals as females, which allows us to compare preferences between the sexes.

Experimental factors: Previous work showed that experimental design impacts mating preference outcomes (Dougherty & Shuker, 2015). Moreover, response to video playbacks compared to live fish varies with at least one of the tested species (Roberts et al., 2017) and could potentially be affected by the sex of the focal individual. We thus included three moderators to reflect the variability in experimental setups. Namely, we included the size of the association zones (5 or 10 cm), the stimulus type (behaving fish, motorised model, fish animation, video playbacks), and recording duration times (5, 10, 15, or 20 min) as experimental factors in our model. We predict that a larger association zone and longer recording duration will result in stronger effect sizes as more data are included. We expect some differences between species due to variations in stimulus type (e.g., live versus video playback).

Phylogeny

To control for the non-independence of the strength of preference due to a shared evolutionary ancestry that varies between species pairs, we included phylogenetic information in our statistical models, using cytochrome b sequences.

Cytochrome b mitochondrial sequences for each species were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank). Such sequences are commonly used as a reasonably neutral marker to determine phylogenetic relationships between species. Genetic distance, representing the amount of time two lineages have been diverging independently, was estimated as the number of base substitutions per site using the Kimura 2-parameter model (Kimura, 1980) in MEGA11 (Tamura et al., 2021). Rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The distance matrix was then converted as a phylogenetic tree using the Maximum likelihood method with Nearest-neighbor interchange (initial tree: NJ/BioNJ).

Statistical analyses

All statistical analyses were carried out in R version 4.2.0 (R Core Team, 2022). We used the package metafor (Viechtbauer, 2010) to perform the meta-analysis modelling. To determine the overall mean effect size, we ran a first multi-level meta-analysis model fitted via restricted maximum likelihood ("REML") estimation with the function rma.mv. We included study identity as a random effect to account for the non-independence of effect sizes. We removed species as an additional random effect as this variable explained 0% of the variance and removing it slightly improved the AIC score of the model. Phylogeny was included in all our

models as a variance-covariance matrix estimated from the phylogenetic tree. To assess the respective influence of our different moderators (i.e. explanatory factors) on the mean effect size, we ran meta-regression models for each moderator separately (function rma.mv with the 'mods' parameter). We calculated the level of heterogeneity across all effect sizes using the I^2 statistic to determine how generalizable our findings are (Higgins et al., 2003). Script for statistical analyses and data are available on OSF: https://osf.io/hnf8m/.

Results

In total, we extracted 62 effect sizes from 15 studies investigating 20 species of darters. This includes 29 effect sizes for males and 33 for females, 42 for allopatric and 20 for sympatric populations.

We found no publication bias in our dataset as shown by a roughly symmetrical funnel plot (figure 2). This was further confirmed by Egger's regression test (Z = 0.6991, p = 0.4845).

Our multi-level meta-analysis model revealed an overall effect size of medium strength (r = 0.3859, p = .0001, CI = 0.2485 – 0.5233), corresponding to positive preferences for conspecifics. A negative effect size would correspond to preferences biased towards heterospecifics, and an effect size not different from 0 to an absence of mate preference. The total heterogeneity across effect sizes (I^2) amounts to 21.18% (17.9% come from the study identity and 3.25% from phylogeny).

Our investigation of the respective influence of our moderators with separate meta-regression models revealed that geographic relationship impacts variation in effect sizes (Q_M = 13.94, p = .0002). We found no overall difference in preference strength between males and females, nor for any of the experimental factors that we included (size of the association zones, stimulus type, and recording duration times). However, given the highly unbalanced sample sizes for those experimental factors, conclusions should be carefully drawn. The results of our meta-regression models are summarized in Table 1.

Post-hoc tests (paired t-tests) on the relationship between effect sizes and geography (allopatric vs sympatric populations) revealed that effect sizes for sympatric populations are bigger than for allopatric populations, for females (t = -2.9452, p = 0.009, mean ES in allopatry = 0.226; mean ES in sympatry = 0.528) but not for males (t = -2.1052, p = 0.057, mean ES in allopatry = 0.256; mean ES in sympatry = 0.48), as illustrated in Figure 3. This indicates that the stronger mate preferences for conspecifics found in sympatric populations are mostly explained by female preferences.

Discussion

Our meta-analysis of 15 datasets encompassing 20 species of darters revealed a positive mean effect size of preference for conspecific mates when using a dichotomous paradigm based on visual cues. This result suggests that most species in our analysis prefer conspecifics of the opposite sex over heterospecifics. Our investigation of potential moderators of the strength of preference showed that only geographic relationships had a significant contribution, whereas neither sex nor any of the three experimental factors

(association zone size, stimulus type, and recording time durations) contributed. When dissecting the effect of the geographic relationship more specifically, we found that sympatric species had stronger preferences for conspecifics than allopatric species did. Moreover, our post hoc statistical tests suggest that sympatric females are the main drivers of that difference, although sympatric males also tend to more strongly prefer conspecific females than allopatric males do.

Sympatric vs allopatric: A case in favor of reinforcement

Stronger mating preferences for conspecifics in sympatric compared to allopatric populations is the classic signature of reinforcement. Our results are aligned with this prediction as we found that sympatric species more strongly preferred conspecific mates. Our meta-analysis further reveals that this difference is important, with a mean effect size in sympatric species that is double that in allopatric species, with no overlap in confidence intervals. This magnitude is consistent with the findings of Coyne & Orr (1989) in *Drosophila* whose estimation of premating isolation for sympatric pairs was at least twice as large as for allopatric pairs. Though many case studies of single species pairs show a signature of reinforcement, with greater divergence in signals (Husemann et al., 2014) or preferences (Höbel & Gerhardt, 2003) in sympatry, comparative studies are critical for establishing whether reinforcement is a general pattern in nature. Our results, therefore, add an important comparative analysis to the literature supporting the prevalence of reinforcement in a group of Vertebrates.

We further found that the greater strength of preference for conspecifics in sympatric populations was significant only for females, with males showing only a tendency for a similar trend. This result is consistent with classical sexual selection theory, which predicts that females will be choosier than males. In this case, with sympatric females choosier than allopatric females, our interpretation is that the cost of heterospecific mating in sympatry is higher for females than for males. Again, this result is consistent with higher reproductive investment by females. It is also consistent with the results of Yukilevich & Peterson (2019), who found the same pattern in sympatric *Drosophila* species, of greater preference for conspecifics in females compared to males.

However, male darters invest considerably in reproduction, with energetic courtship displays, nuptial coloration, and in some species, paternal care (e.g. Kelly et al., 2012; Mendelson et al., 2018). Notably, for the darter species *E. caeruleum* and members of the *Ceasia* (*E. spectabile*) species complex, only males of sympatric species showed greater preference for conspecifics (Moran & Fuller 2018), in contrast to our results. That study used a different experimental design than those in our analysis, one in which fish were not separated by physical barriers. Mate choice was measured as either the amount of time individuals spent pursuing (males) or the number of nose digs toward (females), the opposite sex. Nose digs by females typically precede spawning in those species and have been used as a measure of preference in other studies of darters (Fuller 2003; Williams and Mendelson 2011; Zhou et al. 2015; Zhou and Fuller 2016). Thus, our contrasting results may be due to differences in experimental design, if mate choice trials that prevent physical interaction, as those used in our analysis, facilitate or alter the expression of female preference. Alternatively, species might differ in the processes underlying reinforcement. The costs and benefits of mating with heterospecifics surely vary across taxa, and darters comprise a diverse lineage of fish occupying a variety of habitats and communities.

Methodological/practical considerations for experimental designs

Beyond the biological implications of our results, we also sought to determine whether experimental factors could influence effect sizes when comparing multiple studies. Given our small sample sizes, we can only draw cautious conclusions. However, in terms of the type of stimuli, i.e., whether stimulus fish were live, model (dummy), video playback, or computer animation, we found that it did not affect the strength of preference for conspecific stimuli. This result is an important validation of the use of artificial stimuli in mate choice research (Chouinard-Thuly et al, 2017; Powell & Rosenthal 2016), in that artificial stimuli appear to be as useful as live animals in detecting mate preference. Artificial stimuli allow researchers to replace and reduce the number of live animals in experiments, as encouraged by ethical animal use protocols. Results of Williams & Mendelson (2011) corroborate our finding, showing equivalent responses to live and dummy fish in *Etheostoma zonale* and *E. barrenense.* Roberts et al. (2017) found equivalent responses to live and video playback stimuli in *E. zonale*, but not in *E. barrenense*. Thus the type of stimulus may have some effect on strength of preference, but this appears to be type- and species-specific. We note that most studies in our analysis use live stimuli. Nonetheless, we continue to explore the efficacy of various stimulus types in testing mate preference in darters.

Although we did not expect a strong effect of recording time duration, we found a tendency for longer durations to have smaller effect sizes. It might thus be important to keep the duration of observation short (i.e. under 10 min,) as the expression of preference may begin to taper after a few minutes as the focal individual loses interest. Besides mean durations in association zones, additional measures of fish preference could provide important information. Additional behavioural measures might include the fish's head orientation or line of sight, and pursuit behaviours, to quantify interest in the presented stimuli. For instance, two studies in darters reported glass jabbing behaviour as a measure of a female's mating interest and of a male's aggressive behaviour (Soudry et al., 2020; Williams & Mendelson, 2013). One study also reports the number of times a fish visits an area (Soudry et al., 2020), which could indicate exploratory differences between species or sex that may reflect preference.

Finally, the last experimental factor that varied between studies is the size of the association zones. In their study comparing several pairs of darter species, Mendelson and colleagues (2018) reported results for zone sizes of both 5 and 10 cm. We found no difference between 5 and 10 cm in terms of effect size neither in their study nor in our meta-analysis (data for 5cm was included in the meta-analysis, but including the data for 10cm instead made no difference either). However, effect sizes tended to be larger for wider association zones, which is logical, since a bigger area of the tank can be occupied for a longer period of time. To avoid inflating results with larger association zones, we recommend adjusting the size of the association zone to reflect the visual acuity of the tested species (Caves et al, 2017).

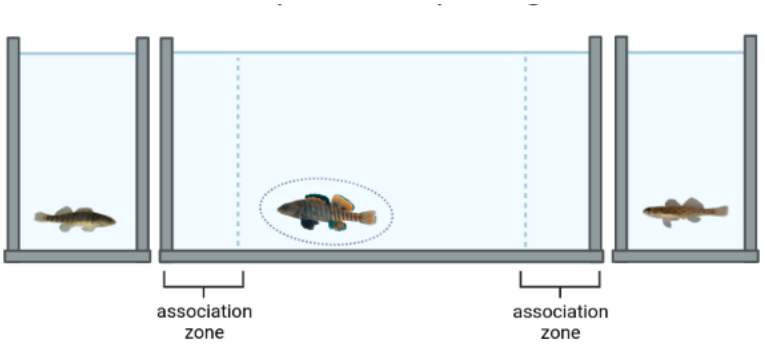
Conclusion

Conducting a meta-analysis of dichotomous mate preference trials in darter fish (*Etheostoma*), we found an overall effect size of medium strength, indicating a preference for conspecific over heterospecific mates, with no difference between males and females.

As for the effect of geographic relationship, we found a stronger preference for conspecific mates in sympatric species pairs, consistent with a hypothesis of reinforcement. In our case, reinforcing selection would appear to translate into greater strength of preference in females, consistent with the idea that mating with a heterospecific is more costly for females than for males. Our results therefore provide a comparative analysis that corroborates case studies of darter species pairs (Moran & Fuller 2018, Roberts & Mendelson 2020), suggesting that reinforcement of preference for conspecific mates is a general pattern in this genus.

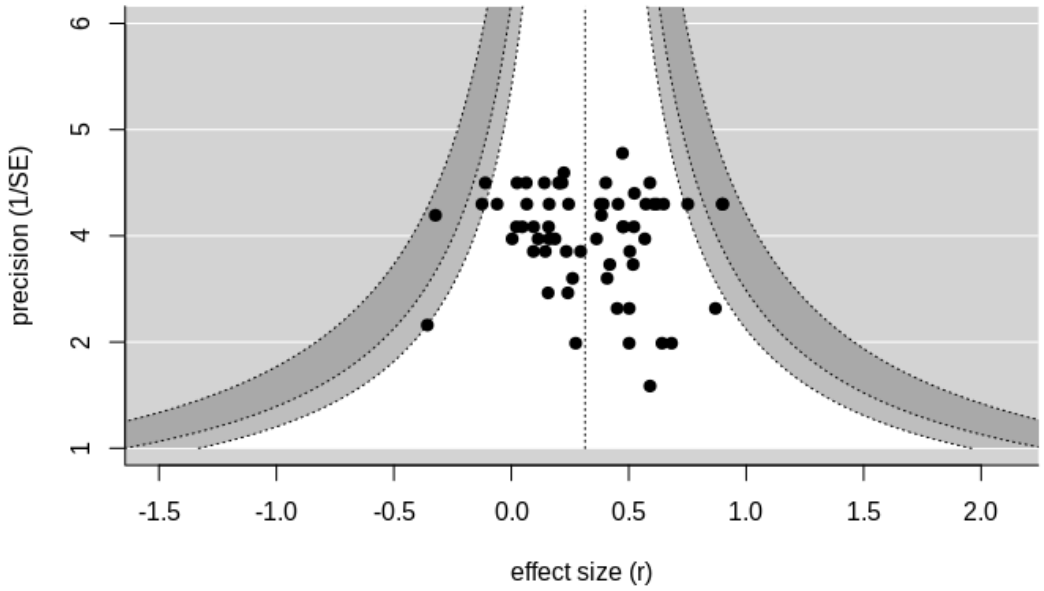
Figures and table

Figure 1: Mate preference experimental paradigm



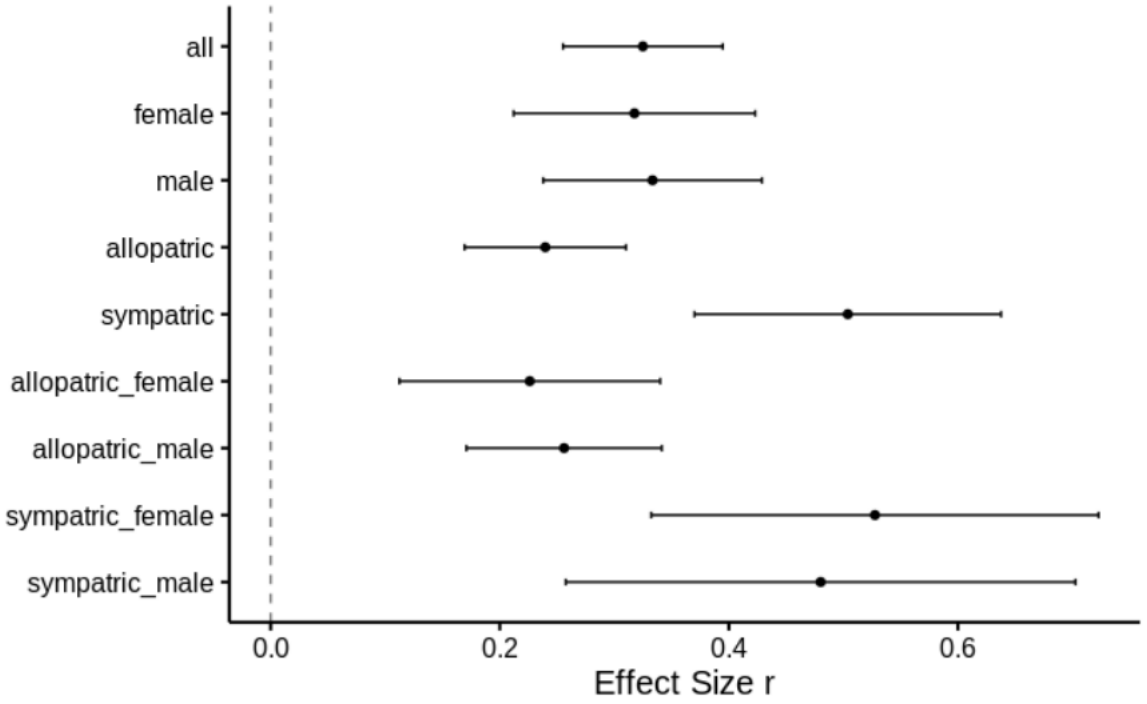
Legend: Illustration of a dichotomous mate preference paradigm. The main measure is the time that the focal fish (circled) spends in either association zone adjacent to either a conspecific or a heterospecific individual of the opposite sex. The exact design varies between studies as mate options can be live fish, motorised models, videos or computer animations displayed on a monitor. Created with BioRender.

Figure 2: Funnel plot to test for publication bias



Legend: The funnel plot is roughly symmetrical, indicating no publication bias, which was confirmed by Egger's regression test: Z = 0.6991, p = 0.4845.

Figure 3: Forest plot of effect sizes



Legend: Forest plot showing mean effect sizes (± CIs) calculated using Equation 1 of the main moderators: sex and geographic relationship, as well as an average of all the moderators included in the analysis.

Table 1: Table with moderators: Q_M, *p*-value, mean, and CIs estimated in separate models for each moderator.

Moderator	Q_M	*p*-value	Mean	Sample size	95% CIs
Sex of the focal individual	0.4954	0.4815	F: 0.3648 M: 0.4176	32 30	0.2137 - 0.5158 0.2593 - 0.5759
Allopatry vs sympatry	**13.9438**	**0.0002**	**A: 0.2252** **S: 0.5434**	**42** **20**	**0.1138 - 0.3366** **0.3810 - 0.7058**
Size of the association zone	0.0875	0.7673	5cm: 0.3722 10cm: 0.4171	53 9	0.2114 - 0.5329 0.1470 - 0.6872
Stimulus type	2.1426	0.5433	Live: 0.3713 Video: 0.2880 Motorised: 0.6001 Animation: 0.3324	53 3 4 2	0.2293 - 0.5134 -0.1100 - 0.6860 0.2878 - 0.9123 -0.2305 - 0.8952
Recording times	0.8924	0.8273	5min: 0.4419 10min: 0.3910 15min: 0.4611 20min: 0.3394	4 1 19 38	-0.0187 - 0.9026 -0.1923 - 0.9743 0.2792 - 0.6429 0.1581 - 0.5208

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The star (*) indicates papers included in the meta-analysis.

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