Preference for conspecific mates in sympatric and allopatric darters (genus *Etheostoma*): comparative evidence for geographical and sex effects

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Abstract

A preference for mating with conspecifics over heterospecifics is fundamental to the maintenance of species diversity in sexually reproducing organisms. This type of assortative mating is often stronger between closely related sympatric species as compared to allopatric species, resulting from processes such as reinforcement or differential fusion. In animals, this pattern has been demonstrated across the genus *Drosophila*, however, most empirical evidence for stronger assortative mating in sympatry 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 21 species. As expected, we found stronger preferences for conspecifics over heterospecifics across studies, species, and sexes. We further found stronger preferences for conspecific mates in sympatric as compared to allopatric species. This was especially true for females, consistent with the idea that mate choice might be more costly for females than males.

Keywords: assortative mating; sexual isolation; reinforcement; Etheostoma; mate preference; speciation

Introduction

As evolutionary biology continues to explore the mechanics of speciation, the processes driving reproductive isolation remain a central focus of study. One important reproductive barrier is sexual isolation, a form of assortative mating resulting from a preference for conspecific mates (Jiang et al., 2013; Kopp et al., 2018). Preference for conspecific over closely related heterospecific mates can evolve as a result of divergent natural or sexual selection in geographically isolated populations, and it can be strengthened in divergent sympatric populations due to processes such as reinforcement (Dobzhansky, 1940; Liou & Price, 1994; Servedio & Noor, 2003). Understanding the evolutionary mechanisms driving the evolution of preference for conspecific mates is an active area of research in the field of speciation.

In 1989, (Coyne & Orr, 1989, 1997) made the important discovery that prezygotic barriers (mate choice) in *Drosophila* were twice as strong in sympatric species than in allopatric species. This geographic pattern was not true for postzygotic barriers (hybrid inviability and hybrid sterility). (Lemmon, 2009) found the same pattern at a smaller scale in two frog species of the genus *Pseudacris*. Females from populations in which the two species were sympatric more strongly preferred conspecific signals, compared with allopatric females, and this preference was stronger when the conspecific signal was the sympatric one (Lemmon, 2009). Comparative studies in plants also support a pattern of greater assortative mating in sympatric species, though Hopkins (2013) points out ways in which those studies could be strengthened.

A geographic pattern of stronger preference for conspecific mates in sympatric compared to allopatric populations can arise for a number of reasons. One is a process of reinforcement when selection against hybrids in sympatry favors increased preference for conspecific mates (Servedio & Noor, 2003). Another is a process of differential fusion, if geographic overlap only occurs when species have accumulated a sufficient degree of conspecific preference prior to secondary contact (Noor, 1999). Although the original interpretation of Coyne and Orr's (1989) results pointed to reinforcement as explaining the pattern of greater sexual isolation in sympatry, more recent analyses suggest that differential fusion and extinction might also contribute to a pattern of stronger sexual isolation in sympatry (Matute & Cooper, 2021). Thus, a stronger preference for conspecific mates in populations that are sympatric with a close congener is an important pattern that can both support and generate hypotheses about the evolutionary mechanisms driving sexual isolation.

Additional studies show a pattern of greater preference for conspecific mates in sympatry when examining one pair of species or populations. For example, (Rundle & Schluter, 1998) found that benthic sympatric females of the threespine stickleback (*Gasterosteus aculeatus)* preferentially mated with their own type, whereas females from allopatric populations showed no preference between males of their own and the other ecotype. 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 contribute to a pattern of stronger preferences in sympatry, but, being limited to single pairs of species or populations, they do not indicate whether the process is general or idiosyncratic. Comparative studies with multiple species are important for elucidating general patterns.

Another important result of broad comparative studies in *Drosophila* is that of (Yukilevich & Peterson, 2019) found that for sympatric species pairs, female *Drosophila* had stronger preferences for conspecific males than males did for females, whereas no sex difference in preference was found in allopatric species. That result is consistent with classic sexual selection theory (Trivers, 1972; Andersson 2004), that females invest more than males in reproduction and thus represent the choosier sex. A greater strength of preference for conspecific mates in females of sympatric populations thus could suggest two things. Mating with a heterospecific in sympatry might be more costly for females than males, leading to reinforcement of females more so than male preferences; or, female preferences evolve faster than male preferences in allopatry, and they are necessary for maintaining species boundaries upon secondary contact.

Here, we focused on darters (genus *Etheostoma*) to perform a comparative analysis of preference for conspecific mates and to explore the role of geography and sex in predicting those preferences. 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, hybridization has been documented in many darter species (Bossu & Near, 2013; Keck & Near 2009), which could provide the substrate for reinforcement, making this clade a good system to examine geographic patterns of mate choice ~~reinforcement~~ at the genus level.

Two studies of darters have tested explicitly for stronger conspecific mate preferences in sympatric populations ~~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 preferences 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*. 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 sexual isolation, in general, remains an open question.

Material and Methods

Inclusion criteria

We conducted our meta-analysis on studies investigating mate preference in a comparable manner in *Etheostoma* 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 21 species of *Etheostoma* distributed across the phylogeny of the genus (Near et al., 2011), 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). Studies in our analysis were conducted by different lead authors in different physical lab spaces but at the same university, using similar protocols. This similarity in design is well suited for meta-analyses, which often suffer from discrepancies in experimental design across studies.

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 $\overline{\mu}_{sample}$ and σ_{sample} correspond to the mean and standard deviation of the time spent with conspecifics ($\overline{\mu}_{sample_C}$) and with heterospecifics ($\overline{\mu}_{sample_H}$), 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 two ‘natural’ factors: geography and sex of the tested individual, and three ‘experimental’ factors: the size of the association zones, stimulus type, and recording duration times.

Geography: 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 Near and colleagues' (2011) phylogeny. Their phylogeny is based on the cytochrome b mitochondrial gene and two nuclear gene sequences, the S7 ribosomal protein (first intron) and the recombination activating gene-1 (RAG1, exon 3). We retrieved the data file (Nexus format) from TreeBase. We pruned the phylogenetic tree with the drop.tip function from the ape R package (Paradis & Schliep, 2019) to keep only our 21 species of interest. In the case where several individuals per species were available, we kept the individual from the closest geographical population to the population studied in our meta-analysis. The resulting tree (Figure 4) was converted into a matrix that was included in our meta-regression models.

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 21 focal 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 ($Z = 0.5529$, $p = 0.5803$).

Our multi-level meta-analysis model revealed an overall effect size of medium strength ($r = 0.4020$, $p < .0001$, CI = 0.2730 – 0.5311), 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 19.46% (16.88% come from the study identity and 2.58% 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 ($Q_M = 12.79$, p = .0003). 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 = -2.9282, p = 0.009, mean ES in allopatry = 0.223; mean ES in sympatry = 0.528) but not for males (t = -2.0371, p = 0.065, mean ES in allopatry = 0.266; 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 21 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 geographic pattern of assortative mating

Our main result is that preferences for conspecific mates are stronger in sympatric compared to allopatric populations. 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 greater divergence in signals (Husemann et al., 2014) or preferences (Pauers & Grudnowski, 2022; Höbel & Gerhardt, 2003; Pauers & Grudnowski, 2022) in sympatry, comparative studies are critical for establishing whether this geographic pattern of mating trait divergence is a general pattern in nature. Our results, therefore, add an important comparative analysis to the literature supporting this geographic pattern.

A pattern of stronger preferences for conspecific mates in populations that are sympatric with close congeners can result from multiple processes. Reinforcement, for example, is the strengthening of conspecific preference due to selection against low-fitness hybrids in sympatry (Dobzhansky, 1940; Servedio & Noor, 2003) and may explain our results. Darters are known to hybridize in nature (Keck & Near, 2009), and hybrid fitness is reduced in many pairs of darter species (Martin & Mendelson, 2018; Mendelson et al., 2006, 2007), potentially supporting a hypothesis of reinforcement. Another process that could explain this pattern is “differential fusion” of lineages upon secondary contact (Noor, 1999). Diverging allopatric lineages that have not evolved strong sexual isolation may fuse upon secondary contact, whereas lineages with strong sexual isolation will persist, leaving a pattern of stronger preferences for conspecifics in sympatric species. Interestingly, however, when secondary contact is simulated in artificial streams for allopatric species of darters in the earliest stages of divergence, the strength of preference for conspecifics is stronger in males than in females (Mendelson et al., 2018), which is not what we found in our meta-analysis. If the geographic pattern in the strength of preference we found was due to differential fusion, we would be more likely to have found stronger preferences in males compared to females.

Rather, we found that the greater strength of preference for conspecifics in sympatric populations and species 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.

A third process that could explain our pattern is “reproductive interference,” where the fitness of adults is negatively affected by interspecific interactions stemming from an incomplete preference for conspecifics, irrespective of hybrid fitness, for example wasting energy on unsuccessful courtship (Gröning & Hochkirch, 2008). Whether due to reinforcement or reproductive interference, our data support a hypothesis of selection for increased preference for conspecifics in females in sympatry.

Yet, 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 & 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 costs and benefits of mating with heterospecifics. These costs 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, 2017), 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 and colleagues (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. We also found a stronger preference for conspecific mates in sympatric compared to allopatric species pairs, and this pattern appears to be driven by female, rather than male preferences. A pattern of increased preference for conspecifics in sympatric populations is consistent with multiple evolutionary processes, including reinforcement, differential fusion, and reproductive interference. Given the evidence of reduced hybrid fitness in many darter species and sex differences in strength of preference consistent with selection after secondary contact, a hypothesis of reinforcement may best explain our results. Our comparative analysis therefore corroborates case studies of darter species pairs (Moran & Fuller, 2018; Roberts & Mendelson, 2020) and suggests that selection for increased preference for conspecific mates in sympatry may be common in this genus.

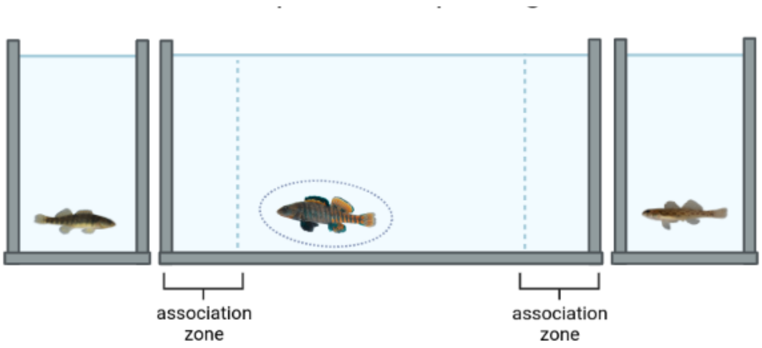
Table

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	0.6783	0.4102	F: 0.3758 M: 0.4378	32 30	0.2316 - 0.5201 0.2858 - 0.5898
Allopatry vs sympatry	**13.5258**	**0.0002**	**A: 0.2419** **S: 0.5493**	**42** **20**	**0.1236 - 0.3602** **0.3825 - 0.7161**
Size of the association zone	0.0184	0.8922	5cm: 0.3977 10cm: 0.4180	53 9	0.2490 - 0.5464 0.1555 - 0.6805
Stimulus type	2.2142	0.5291	Live: 0.3801 Video: 0.2885 Motorised: 0.6010 Animation: 0.3271	53 3 4 2	0.2360 - 0.5242 -0.1044 - 0.6815 0.2943 - 0.9078 -0.2324 - 0.9078
Recording times	0.6456	0.8859	5min: 0.4379 10min: 0.3901 15min: 0.4599 20min: 0.3579	4 1 19 38	-0.0150 - 0.8909 -0.1871 - 0.9674 0.2793 - 0.6406 0.1783 - 0.5375

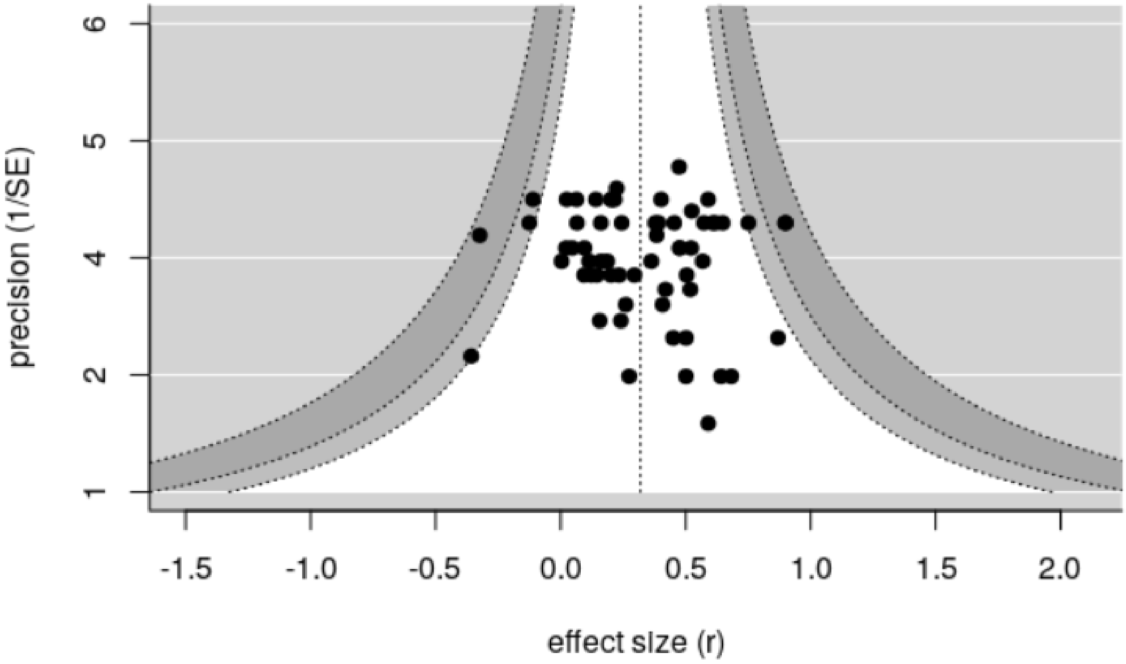
Figures

Figure 1: Mate preference experimental paradigm



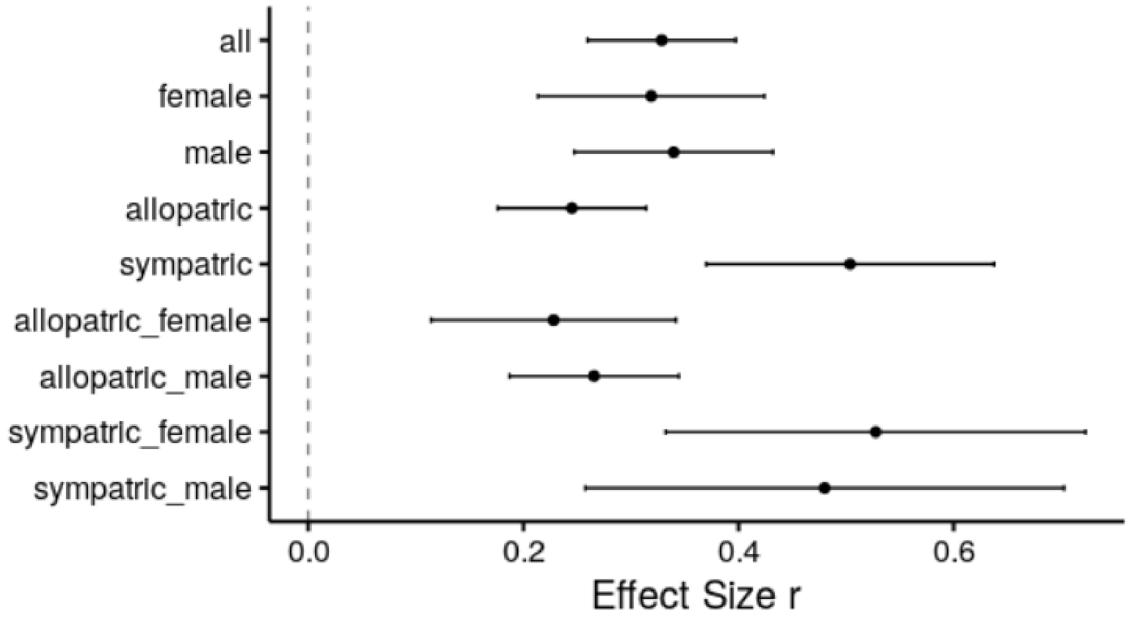
Legend: Illustration of a dichotomous mate preference paradigm. The main measure is the time that the focal fish (circled) spends in either association zone adjacent to either a conspecific or a heterospecific individual of the opposite sex. The exact design varies between studies as mate options can 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



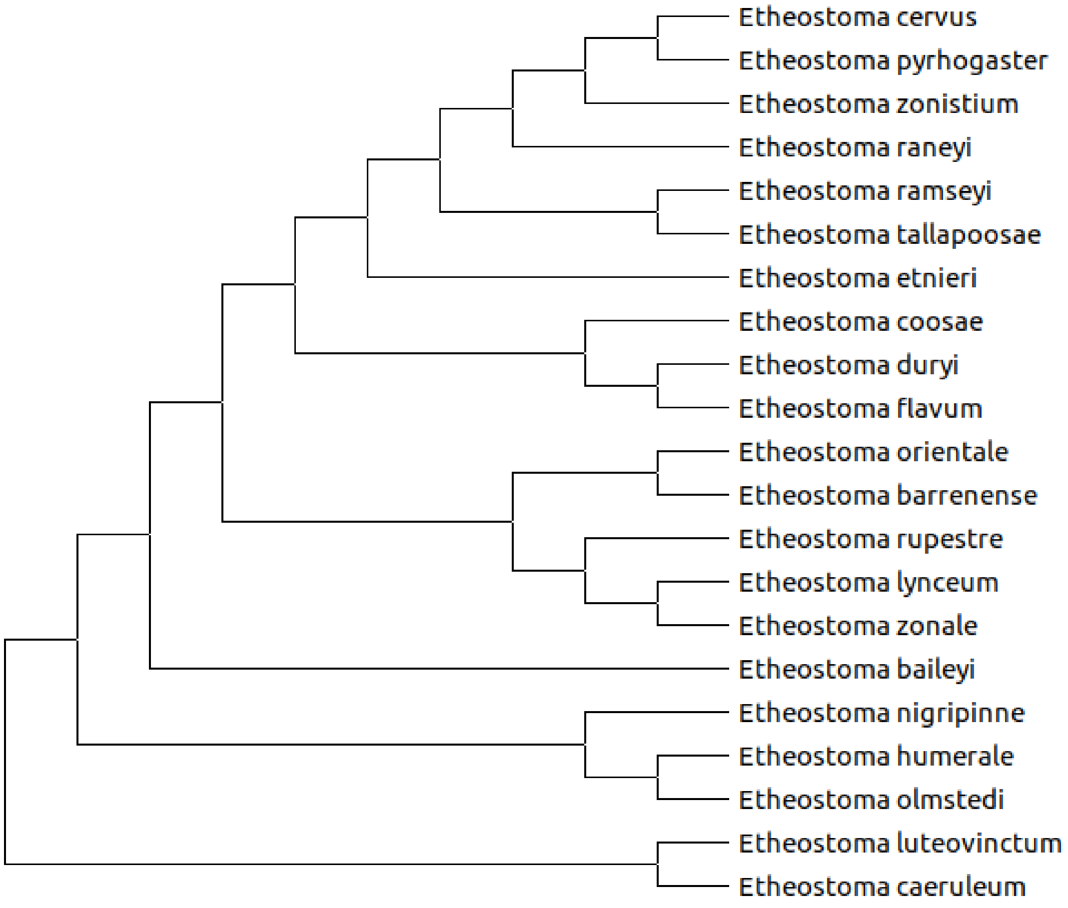
Legend: The funnel plot is roughly symmetrical, indicating no publication bias, which was confirmed by Egger's regression test: Z = 0.5529, p = 0.5803.

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.

Figure 4: Phylogeny of the focal species included in the meta-analysis



Legend: Phylogenetic cladogram of the 21 focal species included in the meta-analysis, based on the phylogeny of Near et al. (2011). Note that E. humerale corresponds to our E. flabellare (collected from the Potomac River, an Atlantic slope drainage).

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

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