



# An integrated approach to infer the mechanisms of mate choice for size

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Size-assortative mating and sexual selection on size are common across species. Since both may be a result of mate choice, mate choice based on size should also be a widespread process. This behaviour is, however, rarely studied directly and thus the biological causes that determine size-based mate choice are poorly understood. To address this, we studied the size-based mate choice in an intertidal snail, *Echinolittorina malaccana*, that has been used as a model to understand this process. Previous studies, assuming a quantitative Gaussian mating preference function, have inferred that mate choice in this snail is caused by a size similarity mechanism (males prefer to mate with females slightly larger than themselves). To further test and quantify this proposed mechanism, we conducted mate choice experiments with alternative designs (single, male and multiple choice) in the laboratory and compared the results to mate choice data observed in natural populations. This integrated approach allowed us to elucidate the mechanism of mate choice by evaluating alternative mating models that best fitted the observed data of various designs. Results confirmed the similarity-based mechanism but showed deviations at extreme size classes. The single choice design indicated that mate choice was exercised during one-on-one male–female interactions, but the strength of mate choice increased with the presence of additional individuals (males in the male choice design, and both males and females in the multiple-choice design). Multiple-choice experiments are, therefore, the most valuable and useful design to infer how males choose mates in the wild, as they best mimic the natural scenario and the results are the most similar to those observed in natural populations. To elucidate the mechanisms causing this mate choice for particular female sizes, the next steps are to identify the genetic basis as well as potential physiological benefits associated with choosing slightly larger females.

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Sexual reproduction is a key process for most organisms, and so choosing a specific sexual partner from the available population is of key evolutionary relevance (Jennions & Petrie, 2000; Roff, 2015). Choice of mates is vitally important, as individuals differ directly in the quality of genes (for survivorship and reproduction), or indirectly by the resources or experience that they have gained during

their lives (Pierotti, Martínez-Fernández, & Seehausen, 2009). As a consequence of these individual differences, to prefer a certain individual as a mate can have important fitness consequences, being the difference between failure or success in the production of viable progeny (Andersson, 1994; Gavrilets, 2004; Müller, Lachenicht, & Müller, 2018). To enhance fitness, individuals often select mates that have certain traits that are assumed to be beneficial (Andersson, 1994; Burley, Hamedani, & Symanski, 2017; but see Boughman, 2001). The behavioural propensity to mate with individuals having certain traits is called mate choice (which here is assumed to be synonymous with mating preference, following Basolo, 1998; Rolán-Alvarez et al., 2015; reviewed in Edward, 2015; Roff, 2015 and references therein), although alternative definitions also exist (Edward, 2015; Gavrilets, 2004, or Rosenthal, 2017). The practical consequence of mate choice is that mating within a

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population is no longer random (Gavrilets, 2004), causing either assortative mating, sexual selection or both effects (Lewontin, Kirk, & Crow, 1968; Rolán-Alvarez & Caballero, 2000). Assortative mating is defined here as the correlation between homologous phenotypes across members of mated pairs (sensu Jiang, Bolnick, & Kirkpatrick, 2013), while sexual selection is the selection differential for the trait between mated and unmated specimens in the population (following the classic definition of Arnold & Wade, 1984). Therefore, in addition to being a key feature for individual fitness, the consequences of mate choice can be a key evolutionary process for populations and indeed the entire species (Jennions & Petrie, 1997). Sexual selection and assortative mating effects can, however, be affected by other processes not associated with mate choice itself, such as mate competition (Taborsky, Guyer, & Taborsky, 2009). Indeed, assortative mating can be biased by nonrandom trait distributions (Crespie, 1989; Taborsky, Guyer, & Demus, 2014) or a result of a decoupling between sampling and mate choice scales (Ng, Williams, Davies, Stafford, & Rolán-Alvarez, 2016; Rolán-Alvarez et al., 2015).

Mate choice and its mechanism have been studied and discussed in many species for several traits (reviewed in Dougherty & Shuker, 2015; Jennions & Petrie, 1997), and specifically in relation to individual size (Ambrogio & Pechenick, 2009; Backwell & Passmore, 1996; Eddy et al., 2016; Kim, Kim, Hong, & Choe, 2006; Luo et al., 2014; Pollo, Muniz, & Santos, 2019; Shine, O'Connor, Lemaster, & Mason, 2001; Taborsky et al., 2009; Tejada, Tejada, Arredondo, Díaz-Fleischer, & Pérez-Staples, 2020; Yu & Wang, 2013), including in humans (Tenesa, Rawlik, Navarro, & Canela-Xandri, 2015 and references there in). The effect of size on mate choice can be complex, as size may be used as a criterion for an individual to choose a mate (for example when an individual prefers to mate with a larger partner) or influence the rules of choice of an individual (for example individuals of different sizes may have different preferences). However, in any similarity-like mate choice mechanism the two former possibilities are equivalent (see below).

In general, mate choice for different traits/types has been investigated in the laboratory following four main experimental designs (e.g. Coyne, Elwyn, & Rolán-Alvarez, 2005): no choice (termed single choice hereafter), male choice, female choice and multiple choice. Single choice is when one male and one female of each type combination are placed separately in the experimental mating chambers. This design is statistically robust as the data obtained are truly independent (Noor & Ortiz-Barrientos, 2006). However, with this approach, intrasexual competition cannot be estimated and even intersexual mate choice is considerably simplified (as only mechanisms that affect that particular male and female type are possible, for example, Knoppien, 1985), and mate choice is weaker under no-choice designs than under choice designs (Dougherty & Shuker, 2015). A male choice design makes some attempt to address these issues when an isolated male is placed with different female types. Even under such a design, only competition among females is possible and, again, the mechanisms of mate choice are over simplified (although the male could show preference for any of the female types). A female choice design is similar but placing an isolated female type with different male types, and so suffers from the same limitations. A multiple-choice design overcomes many of these drawbacks as it can be used to study mate choice and competition in both sexes. In principle, this design represents a more accurate reflection of nature, at least for those species in which multiple males and females meet simultaneously (Alipaz, Fang, Osada, & Wu, 2005; Spieth & Ringo, 1983) and allows all interactions within and between sexes. Certain multiple-choice designs have, however, been criticized because the different mating pairs observed may be statistically dependent (Noor & Ortiz-Barrientos, 2006).

Alternatively, mate choice can be directly estimated in the natural environment in those species that are common (and feasible) to find and capture (or measure) mating pairs. The possibility of obtaining a set of representative mating pairs from a natural population allows the indirect inference of mate choice by measuring its consequences (such as estimating the strength of assortative mating, Kim et al., 2006; Ng, Williams, et al., 2016, 2019b; Rolán-Alvarez et al., 2015; Taborsky et al., 2009). Even in these classic field studies, any assortative mating observed can, however, be biased or even caused by other processes (Crespie 1989; Indykiewicz et al., 2017; Ng, Williams, et al., 2016; Rolán-Alvarez et al., 2015; Taborsky et al., 2014). In addition, due to the difficulties in achieving adequate sample sizes and developing robust estimators, relatively few studies have tried to estimate mate choice directly from observed mating pair frequencies (e.g. Estévez et al., 2018; Fernández-Meirama, Carvajal-Rodríguez, & Rolán-Alvarez, 2017b; Luo et al., 2014). This direct estimation process is also potentially biased, as mate choice can be partially confounded with the trait frequency in the population (see further discussion in Fernández-Meirama, Estévez, et al., 2017). Estimates based on natural observations are, therefore, also prone to biases and the ideal approach to test for possible mate choice is a combination of field-based observations and controlled laboratory experiments.

Gastropods share common characteristics in their reproduction, as they are typically gonochoric and polygamous, and have internal fertilization (Ng et al., 2019b). Intertidal gastropods also have the benefit of being relatively easy to observe mating behaviours on the shore and, as a result, several studies have estimated size-assortative mating and sexual selection for size in this group (reviewed in Jiang et al., 2013; Ng et al., 2019b), which show positive size-assortative mating and sexual selection favouring larger females coupled with sexual size dimorphism (females being slightly larger than males). These patterns have been explained by a mechanism of mating preference by similarity (with individuals having a preference for similar-sized partners), but with a bias towards larger sizes (for example a male of size X would prefer females of size X + B, B being the bias of this similarity preference; see Ng et al., 2019b).

*Echinolittorina malaccana* is a high-shore gastropod living on rocky shores across the Indo-West Pacific region (Reid, 2007). Males search for female mates by following their mucus trails and when mounting the female, they decide to mate or not based on tactile interactions that are difficult to quantify or observe. In this process, females mostly play a passive role and have only rarely been observed to reject mating by pushing the male away (Ng et al., 2019b). The main mechanism of mate choice in this species, as well as a number of other littorinid species, appears to be that males show a preference for females somewhat larger than themselves (Ng et al., 2019b). Specifically, *E. malaccana* has been demonstrated to show mate choice based on a similarity plus a bias mechanism (López-Cortegano, Carpena-Catoira, Carvajal-Rodríguez, & Rolán-Alvarez, 2020). The estimated strength of the mate choice, assuming a Gaussian preference function, is of intermediate value (able to cause a size correlation in pairs of about 0.5) while the bias in the male preference was estimated to be about approximately 6% of the male size (i.e. males prefer to mate, on average, with females 6% bigger than themselves; López-Cortegano et al., 2020). These estimates represent the only approximation available for a quantitative trait like size, although, as noted above, these are still potentially biased. To overcome these issues, and to confirm whether these parameters are the only relevant ones acting on size selection, alternative mate choice experiments in the laboratory are required for the corroboration of these estimates as well as the proposed mechanism.

To address these issues, we investigated mate choice based on size in *E. malaccana* combining laboratory experiments with onshore observations. To conduct an inferential test, we transformed size (a continuous trait) into a qualitative trait (discrete size classes), which allows the use of classic mate choice experimental designs and the comparison of these alternative mate choice models by a model selection methodology (see Carvajal-Rodríguez, 2020). This application of complementary approaches allows us to disentangle the mechanism contributing to mate choice on size and, therefore, provide a robust estimation of the contributing parameters.

## METHODS

### Laboratory Mate Choice Experiments on Size

Snails were collected from Cape d'Aguilar rocky shore (22°12'33"N and 114°15'28"E) in July 2018 and in August 2019, as most mating and spawning occur from June to September in Hong Kong in this species (Mak, 1998; Ng, Davies, Stafford, & Williams, 2016). Individuals were transported to the laboratory, sexed and maintained in dry conditions prior to the experiments (see Ng et al., 2019b). As these high-shore snails frequently experience days without tidal inundation, the relatively short maintenance (a few days) prior to the experiments was assumed to match normal conditions. All snails were returned alive to the shore after experiments.

Individuals were sexed and divided a posteriori into four nonoverlapping size classes based on shell length: size class S1 (4.0–4.9 mm), S2 (6.0–6.9 mm), S3 (8.0–8.9 mm) and S4 (10.0–10.9 mm). As the snails from different size classes differed by 1–3 mm in shell length, they could be distinguished by eye and thus no tagging was required. Snails were kept dry prior to the experiments to mimic low-tide conditions, so that when they were wetted they would become active and enter the mating phase as would occur on the shore with the rising tides (Ng et al., 2019b). While the multiple-choice design represents the most realistic situation (as potential mates for *E. malaccana* are abundant on the shore and there is a high conspecific density), using complementary, alternative mate choice designs allows us to disentangle the mechanisms of mate choice (Coyne et al., 2005; Dougherty & Shuker, 2015), especially when compared with data collected on the shore. For example, in *E. malaccana* mate choice starts when the male follows a female mucus trail (Ng et al., 2016a, 2019b), and so

using a male choice design will capture a relevant part, but not all, of the biological mechanism involved in mate choice, and so a comparison of multiple- versus male choice designs will identify the processes that occur when males simultaneously compete for mates. Finally, a single-choice design would also allow the identification of the basic mechanism of choice when isolated male and females are interacting.

Given these considerations, we therefore conducted multiple-choice (Multiple; males and females from all four size classes in the same chamber), male choice (Male 1 and Male 2) and single-choice (Single; one male and one female) experiments (Fig. 1). Male 1 was a typical male choice design (one male with females of four different sizes), whereas for Male 2, 1 h before the experiment males of other size classes were maintained in the chamber and then removed before the experiment started. This design allowed us to assess the relative importance of the chemical presence (the mucus trails) of other males (if Male 2 showed a similar trend to Multiple) and the physical presence of other males (if Male 2 showed a similar trend to Male 1) in influencing mate choice. Multiple and Male designs were conducted in large, experimental mating chambers (plastic transparent spheres, 12 cm diameter), while the Single design was conducted in smaller chambers (8 cm diameter; Fig. 1). Once a complete set of snails were placed in an experimental trial, the experiment ran for at least 4 h.

Mating chambers (transparent plastic spheres; Fig. 1) were sprayed with sea water before the experiment to provide a moist environment. Individual snails were immersed in sea water to stimulate foot extension prior to being transferred to a fixed starting point in the mating chamber. If a snail failed to gain foot attachment within 5 min of transfer, it was considered inactive, removed and replaced by another individual. Females were always introduced first and the order of the different experimental treatments (male or female size classes) was randomized for every experiment. Snails were introduced one by one, typically with a 1 min delay, and all individuals came into contact with the mucus trails laid by previously introduced snails.

### Onshore Data: A Reanalysis

Data from mating pairs captured on the shore for several populations of *E. malaccana* are available from Ng et al. (2019a and 2019b). In summary, we searched for mating pairs as the tide rose and the snails became awash when snails of both sexes became wet and started to move, engaging in foraging and reproductive

(a)

Single:		S1	S2	S3	S4
S1	1+1	1+1	1+1	1+1	1+1
S2	1+1	1+1	1+1	1+1	1+1
S3	1+1	1+1	1+1	1+1	1+1
S4	1+1	1+1	1+1	1+1	1+1

Male 1–2:		S1	S2	S3	S4
S1–S4	1+4	1+4	1+4	1+4	1+4

Multiple:		S1–S4
S1–S4	4+4	



**Figure 1.** (a) The experimental designs. Single-choice design (Single) consisted of one male and one female in each mating chamber (i.e. with 16 different combinations of four size classes, S1–S4). Male size classes in columns and female size classes in rows. Male choice designs (Male 1 and Male 2) consisted of one male and four females in each mating chamber (i.e. four combinations). Male 2 was a modified male choice design, with males of the other three size classes introduced for some time and then removed prior to the start of the trial (see text). The Multiple design consisted of four males and four females in each mating chamber (i.e. one combination). (b) View of a single trial with 16 small chambers for the single-choice experiments, four large chambers for the male choice design and one large chamber for the multiple-choice chamber.

activities. Once a mating pair was identified both individuals were captured (together with two to four of the closest, unmated individuals) and brought back to the laboratory where their size (shell length) and sex were recorded (see further details in Ng et al., 2019b). As these studies recorded the size of every individual, we were able to reclassify the data a posteriori into four qualitative size classes to compare the onshore data with our laboratory experiments. To maximize replication these size classes, in contrast to the laboratory data set, were recoded without any size difference between the classes (S1: minimum to 5.9 mm; S2: 6.0–7.9 mm; S3: 8.0–9.9 mm; S4: 10.0 mm to maximum).

### Statistical Analyses

In the laboratory mate choice experiments and the onshore data, each mating pair was obtained from an independent experimental trial (or independently captured; hereafter referred to as nonrepeated mating data). However, in the laboratory mate choice experiments occasionally more than one mating pair could be obtained from an individual mating chamber, violating the assumption of independence of the data, but increasing sample size. These data were included with the original nonrepeated mating data and are, hereafter, referred to as repeated mating data. To determine whether the repeated data could be used without changing the overall trend, nonrepeated versus repeated mating frequency data were compared using homogeneity  $G$  likelihood tests (Sokal & Rohlf, 1995). Summarized data from the 2018 and 2019 onshore observations were similarly evaluated. Mating frequency data were analysed by a statistical partition of  $G$  likelihood tests to detect sexual isolation and sexual selection effects by the software JMATING 1.0 (Carvajal-Rodríguez & Rolán-Alvarez, 2006).

Mating frequency data were analysed using a new size categorization (hereafter differential size classes). As size classes differed, on average, by the same shell length (2 mm), under this approach, a mating pair of an S1 male and an S2 female could be considered equivalent to a mating pair of an S2 male and an S3 female (as they differed, on average, by the same magnitude). Therefore, the 16 combinations of possible mating pairs (a  $4 \times 4$  mating frequency table; S1 to S4 males  $\times$  S1 to S4 females) could be grouped into seven linear classes (-6; -4; -2; 0; 2; 4; 6; describing the mean size difference between the male and the female in mm). Random mating was, therefore, tested by a likelihood goodness-of-fit  $G$  test, using the frequency of different combinations of mating pairs grouped into these seven classes and compared with the expected distribution under random mating. This approach can indicate whether a systematic bias exists (i.e. if males show a higher mating frequency for females larger than themselves). This method assumes that mate choice works in a similar fashion to a Gaussian mating preference function and shows the same trend across the whole size range (López-Cortegano et al., 2020). A more realistic approach can be accomplished by estimating the best fit mating models (see below). Frequency data were analysed by PopTools (Hood, 2010) and SPSS 24.0 version (SPSS Inc., Chicago, IL, U.S.A.).

### Estimating the Mate Choice Mechanism from Model Selection

Any particular observed mating patterns (observed frequency of different mating pair combinations), either from the laboratory mate choice experiments or from onshore observations, can be caused by many different biological mechanisms (hereafter referred to as mating models). In particular, our mating models can be determined by two main kinds of parameters (see Supplementary Table S3): the mate choice parameters, choice ( $C$ ) and choice bias ( $B$ ), which determine the frequency of mates in one or a few combinations of mating pairs; and the mate competition

parameters,  $a$  and  $d$ , which determine the mates for some female (row) or male (column) size classes. These mate choice and mate competition parameters represent the classic forces modelled in previous studies investigating the evolution of assortative mating and sexual selection (see Carvajal-Rodríguez, 2018; O'Donald, 1980). The mate choice and mate competition parameters can be estimated from the  $4 \times 4$  mating frequency table in several ways (Table S3). We performed model selection and parameter estimation using InfoMating version 0.4 (Carvajal-Rodríguez, 2020). Implicitly, all models can be considered probabilistic models (sensu Roff, 2015) as the parameters assume certain tendencies for a particular class, but also consider the possibility of individual variation in choice at any moment.

The model selection and parameter estimation procedures are as follows: for each model in a predefined set of models (Table S3), for example B-7P, the maximum likelihood estimates of the parameter values are obtained and used to compute the expected frequencies of mating pairs (Carvajal-Rodríguez, 2018, 2020 and, for clarity, all abbreviations used in the paper follow those used in the software package, InfoMating version 0.4). The particular fit of these expectations with the observed mating frequencies is quantified by the Akaike information criterion corrected for low sample size (AICc) and all the models in the set are ranked from the best to worst fit. When the best model showed a poor fit, we used multi-model inference (Burnham, Anderson, & Huyvaert, 2011), which infers the parameters from the weighted average of a subset of the best models (Carvajal-Rodríguez, 2020).

### Ethical Note

All individuals were sampled from a nonendangered high-density population and collected with corresponding permission of local authorities (Permit No (18) in AF GR MPA 08/9 Pt.3 and (58) in AF GR MPA 08/9 pt.5, from The Agriculture, Fisheries and Conservation Department of the HK SAR Government). All individuals used in laboratory experiments or field work observations were captured, transported to the laboratory, used in mate choice trials (in the experiments), measured for size and sex determined, and later returned alive to the same location.

## RESULTS

During the experiments, trail following was observed in both males and females at certain times in all designs of experiments (Single, Male and Multiple). Male–female behavioural interactions were observed either in the form of mucus trail following or direct contact. Males trail following females did not necessarily lead to mating (as the male might mount the female but then leave the female without mating), whereas males encountering females without prior trail following may sometimes result in mating. As it is unlikely that any two snails in a chamber will not encounter each other (either chemically in terms of detecting the mucus trails of one another or physically in terms of moving into each other), the absence of mating pairs in our study was interpreted as a choice of the male not to mate, instead of the male not detecting the presence of these potential mates. In addition, no females were observed to actively reject males by pushing them away, as they very occasionally do on the shore, and no male–male competitive interactions were observed. Males did, however, appear to vary in their fidelity to their mates (some mated with multiple females, whereas some ignored other females and repeatedly mated with only one female). In many of the trials no mating pairs were observed (see Table 1), but such a high rate of 'null' data is within expectations for mating experiments of marine snails (e.g. Rolán-

**Table 1**

Description of data collection and basic frequency tests under different experimental designs (Single, Male 1, Male 2 and Multiple) and onshore observations (Wild)

Design	Year	N		Homogeneity G test G (df)		G partition in JMATING G (df)		Size difference
		Population	Mated	Between years	Nonrepeated vs repeated mating	Assortative mating	Sexual selection	$\chi^2$ value (df)
Single	2018/2019	357	86	24.7* (11)	8.1 (11)	36.8*** (9)	42.4*** (6)	217.3*** (6)
Male 1	2018/2019	289	48	7.7 (12)	2.1 (12)	12.3 (9)	34*** (6)	164.9*** (6)
Male 2	2019	225	49	–	0.4 (8)	5.2 (9)	58.6*** (6)	109.5*** (6)
Multiple	2018/2019	176	35	5.2 (7)	0.6 (8)	13.2* (9)	46.2*** (6)	157.4*** (6)
Wild	2012/2015 <sup>a</sup>	876	228	–	–	67*** (9)	51.8*** (6)	2146.7*** (6)

Population sample size, *N*, represents the experimental sample size (or unmated specimens in Wild data), while Mated *N* represents the mating pairs observed. The homogeneity *G* test was used to test the difference between years and between nonrepeated and repeated mating data. Assortative mating and sexual selection represent the *G* test partition analyses carried out on every mating frequency table provided in JMATING 1.0 (Carvajal-Rodríguez & Rolán-Alvarez, 2006). Size difference represents the analyses carried out on the seven classes of average size differences within the mating pairs (see Fig. 2 for a representation of the observed and expected frequencies of the different classes).

\*  $P \leq 0.05$ ; \*\*\*  $P \leq 0.001$ .

<sup>a</sup> Data from Ng et al. (2018).

Alvarez et al., 2004). As a result, the observed frequency of mating pairs was used to infer the mechanism of mate choice.

Owing to the low number of snails of different size classes (mostly the extreme size classes) sampled in 2018, a number of Multiple trials were conducted with an incomplete design (seven trials used three males and four females excluding males of the largest size class, and one trial used four males and three females with the absence of females of the smallest size class) which may potentially compromise the analysis of the Multiple data in 2018. No significant difference was, however, found between the raw data of Multiple experiments between years, suggesting that the overall mate choice patterns were not affected by these incomplete trials (Table 1) and analyses including and excluding the 11 mating pairs from these incomplete trials produced similar results. Given the similarity in comparisons and to maximize the sample size for the mate choice model analyses, the mating pairs from the incomplete trials were, therefore, included in all analyses.

#### Sexual Selection and Assortative Mating Analyses

Mating data were homogeneous for nonrepeated and repeated data sets and between years (Table 1) and so were pooled. The Single design was an exception (Table 1), where the experimental effort was unbalanced in 2018, but as different years for Single design data showed similar trends in relation to the mating models (not shown) they were also pooled. All designs showed significant effects of sexual selection and assortative mating, except for Male designs which did not show significant assortative mating effects (Table 1). By contrast, Multiple design data showed clear significant trends in both sexual selection and assortative mating, despite having the lowest sample size, probably because under these conditions the strength of mate choice is maximal.

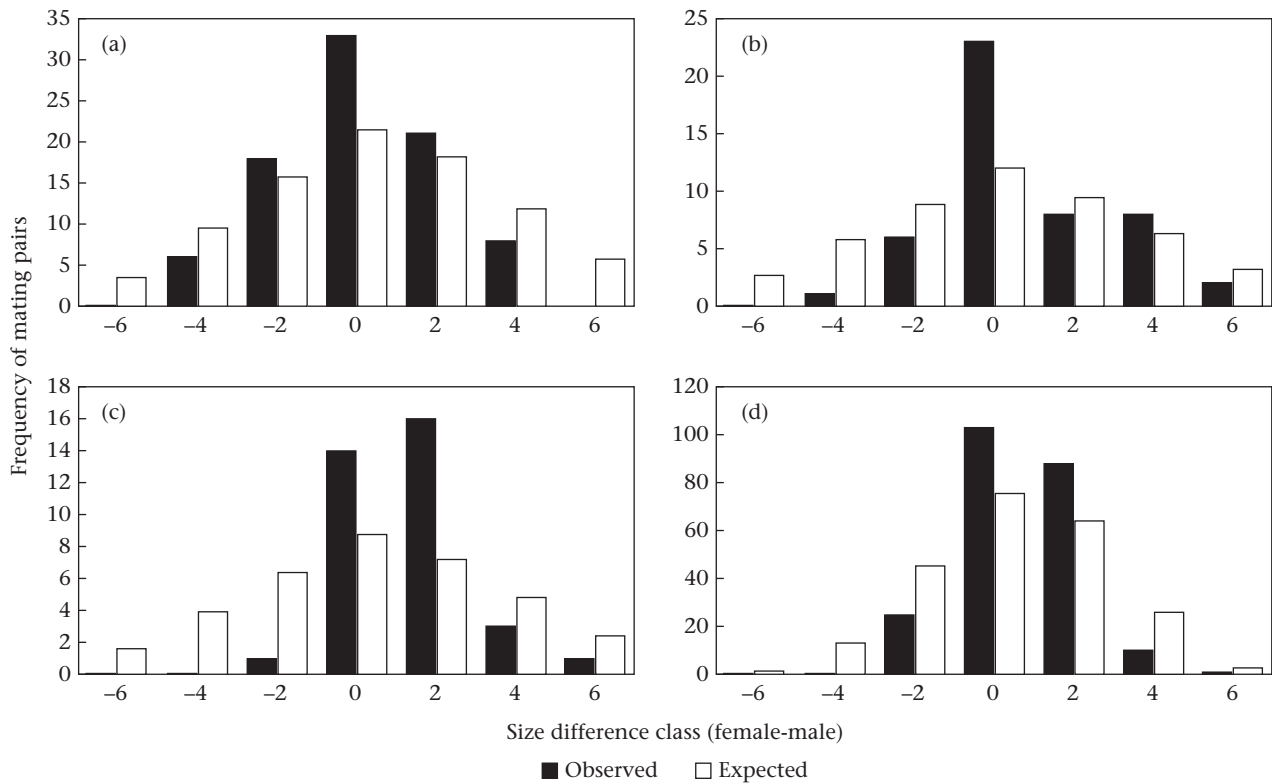
The observed mating data for different classes of size difference did not conform to random mating, with class 0 deviating more from random mating than the other six classes (Table 1, Fig. 2). An excess of observed mating from class 0 can be interpreted as a classic (positive) assortative mating trend, as males of a particular size class showed preference to mate with females of similar size (Fig. 2). Only the Multiple and Wild (i.e. onshore) designs clearly showed an excess of observed mating of class 2, indicating that males preferred females of slightly larger size than themselves (Fig. 2). An estimation of the weighted average of the observed size differences (which represents the size bias in the larger females preferred by the males) was  $0.74 \pm 1.54$  mm in the Wild design, while in the Multiple design it was  $1.37 \pm 1.59$  mm, although this bias varied depending on the specific male size class (see below).

#### Mating Models

Model selection determined which of the possible models (Table S3) best fitted the observed data for different experimental designs (Table 2, Fig. 3). For both the Wild and Multiple designs, the Bias-7P model scored a near perfect weight (close to 100%) and showed four different choice (*C*) and three different bias (*B*) parameters for the different combinations of mating pairs (Table 2, Fig. 3). If we express the resulting model, constructed with the corresponding parameters, relative to one cell (the 2 x 2 size mating class for example) then all the matings can be given in terms of  $C_{22}$  (Fig. 4). Expressed in this way both the Wild and Multiple designs showed similarity-like mate choice (although differing in the strength of choice), which is the preference to mate with similarly sized females, as indicated by the values along the diagonal (Fig. 4). This preference did not, however, occur for the smallest size class (cell 1,1 in the Multiple table from Fig. 4) and was greater for intermediate size classes (cells 2,2 and 3,3 in Fig. 4, larger in Multiple than Wild). There was also a positive mate choice bias, which is the preference to mate with females of the next larger size class, as indicated by the values below the diagonal (Fig. 4). Similar to mate choice, the mate choice bias appeared to be larger (e.g. between cells 3,2 and 4,3 between Multiple and Wild in Fig. 4). If the smallest size classes are excluded, Multiple and Wild models are rather similar, basically differing in the strength of choice, especially for intermediate size classes (Fig. 4).

The Male 1 and Male 2 designs shared the same best fit mating model (SfemC-2Pc), which suggests that, at least in the chamber, the past presence of other males (i.e. the mucus trails placed 1 h in advance) did not influence the subsequent mate choice of the isolated males. However, the best fit model for the Male designs had a low weight (27–30%) which suggests that the mechanism of these mating behaviours is more complex than that explained by the best fit model. Using the multimodel inference approach, both Male designs showed a similar pattern of positive mate choice ( $C \approx 2.5$  and  $C \approx 2$ , respectively, for Male 1 and Male 2 designs; Fig. 4). Like the Wild and Multiple designs, this preference to mate with similarly sized females did not occur at the smallest size class (cell 1,1 in Fig. 4). In contrast, Male designs did not show mate choice bias since the male preference for females of the next higher size class was lower than those of the same size class (subdiagonal values lower than diagonal values in Male 1 and 2 from Fig. 4). Again, if the smallest size classes are excluded, Male 1 and 2 models are basically the same (Fig. 4).

The best fit mating model for the Single design (Smale-2Pc) was similar to that of the Male designs, but with a shift in the position of



**Figure 2.** Distribution of observed and expected frequencies of the seven classes of mean size difference within the mating pairs (i.e. female minus male size; -6; -4; -2; 0; 2; 4; 6 mm; see text). (a) Single-choice design, (b) male choice design, (c) multiple-choice design and (d) the natural onshore data.

**Table 2**  
Analyses of the mating multimodel inference models and parameter estimation

Design	Model ( $\geq 5\%$ weight)	% explained	Parameters in the best model		
			$a$ (female) $d$ (male)	C	B
Single	<b>SmaleC-2Pc</b>	34	$0.1^{***} \pm 0.001$	$1.8^{***} \pm 0.246$	
	SmaleCA-4P	26			
	SmaleC-2P	21			
	SmaleC-4P	8			
Male 1	<b>SfemC-2Pc</b>	30	$0.1^* \pm 0.139$	$2.8^{***} \pm 0.435$	
	SfemC-2P	26			
	SfemC-4P	9			
	SmaleC-2P	7			
	C-2P	6			
	SmaleC-2Pc	5			
	SfemC-4Pc	5			
Male 2	<b>SfemC-2Pc</b>	27	$0^{***} \pm 0.001$	$2.1^{***} \pm 0.353$	
	<b>SfemC-2P</b>	27			
	SmaleC-2P	15			
	SmaleC-2Pc	15			
	SfemCA-4P	6			
Multiple	<b>Bias-7P</b>	99			$C_1:$ $0^{***} \pm 0.002$ $C_2:$ $3.2 \pm 2.189$ $C_3:$ $9.6^* \pm 3.554$ $C_4:$ $13.7^* \pm 5.109$ $B_1:$ $0^{***} \pm 0.001$ $B_2:$ $9.6^* \pm 3.554$ $B_3:$ $15.9^{***} \pm 4.260$
Wild	<b>Bias-7P</b>	99			$C_1:$ $0^{***} \pm 0.004$ $C_2:$ $2.3^{**} \pm 0.388$ $C_3:$ $2.8^{***} \pm 0.380$ $C_4:$ $11.7^{***} \pm 2.703$ $B_1:$ $1.8 \pm 1.029$ $B_2:$ $2.4^{***} \pm 0.342$ $B_3:$ $5.4^{***} \pm 0.740$

Parameters are named as in InfoMating version 0.4 (see Methods). The best fit models are in bold, and % explained and parameters estimated (mean  $\pm$  SD) are given for the different experimental designs. Significance of parameters was evaluated by a Z test (against the null hypothesis that the parameter is 1).

\*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$ .

the mate propensity ( $a/d$ ) parameter and a lower estimate of the C parameter (Table 2, Fig. 3). In the Single design, males of the smallest size class were less likely to mate, while this is also true for

females of the smallest size class in the Male design (Fig. 3). The pattern resulting from the model inference approach was similar to the Male designs, with positive mate choice ( $C \approx 1.5$ ; Fig. 4). Similar

Single (SmaleC-2Pc)	aC	1	1	1
	a	C	1	1
	a	1	C	1
	a	1	1	C
Male 1 Male 2 (SfemC-2Pc)	aC	a	a	a
	1	C	1	1
	1	1	C	1
Multiple Wild (Bias-7P)	C <sub>1</sub>	1	1	1
	B <sub>1</sub>	C <sub>2</sub>	1	1
	1	B <sub>2</sub>	C <sub>3</sub>	1
	1	1	B <sub>3</sub>	C <sub>4</sub>

**Figure 3.** The best fit mating models and the parameters estimated for each combination of mating pairs (see Table 2 for details) for the different experimental designs (Single, Male 1, Male 2 and Multiple) and the onshore data (Wild). The formal names of the mating models are as used in the InfoMating version 0.4 software and are given in parentheses (see Methods and Supplementary Table S3). Male size classes in columns and female ones in rows.

to the other designs, mate choice did not occur for the smallest size class (cell 1,1 in Fig. 4) and, similar to the Male designs, the Single design did not show mate choice bias (subdiagonal values in Fig. 4).

Z tests were used as an independent check of whether the estimation of C and B parameters differed within and between the different designs (Table 2). Some of the C parameters differed significantly between Single and Male 1 designs ( $Z = 2.37$ ,  $P < 0.001$ ) and between Multiple and Wild designs ( $Z_{C2} = 5.13$ ,  $P < 0.0001$ ;  $Z_{C3} = 3.38$ ,  $P = 0.048$ ). Within the Multiple and Wild designs, C<sub>1</sub> differed from all other C estimates ( $P < 0.001$ ) and

within the Wild design C<sub>3</sub> differed significantly from C<sub>4</sub> ( $Z = 6.18$ ,  $P < 0.0001$ ). The remaining comparisons were not significant and, therefore, these trends confirm the parameter differences described in Fig. 4. The B parameters were all significantly different ( $P < 0.001$ ), except between B<sub>2</sub> and B<sub>3</sub> within the Multiple design, and between B<sub>1</sub> and B<sub>2</sub> within the Wild design.

**DISCUSSION**

Details of the mate choice mechanisms in the majority of species remain poorly understood (Dougherty & Shuker, 2015). The present laboratory and field-based experiments attempted to unravel and provide evidence for the rules of mate choice based on size in a species that typically shows size-assortative mating (Ng et al., 2016) and sexual selection on size (Ng et al., 2019b). Mating patterns from all designs of the mate choice experiments in *E. malaccana* deviated from random mating, which clearly indicates sexual selection effects and, in some of the designs, assortative mating was also observed. Assortative mating was not detected under a male choice design, possibly because of the reduced strength of mate choice as well as the low sample sizes, but both the single-choice and multiple-choice designs showed positive assortative mating similar to that measured in the natural populations on the shore (Ng et al., 2016b, 2019b). Positive size-assortative mating and sexual selection favouring larger sizes is a common trend in many species from invertebrates to vertebrates (reviewed in Andersson, 1994; Jiang et al., 2013). For example, in the Asiatic toad, *Bufo gargarizans*, male competition favours larger males, while male–female interactions result in a trend for positive size-assortative mating (Luo et al., 2014). While examples of such patterns are common, the mechanism driving the pattern has only been assessed in a few of these studies.

*Mechanisms of Mate Choice*

Recently, using a quantitative approach and a Gaussian mating preference function, we estimated the strength of mate choice of *E. malaccana* based on two parameters, choice (C:  $0.5 \pm 0.065$  mm) and choice bias (B:  $0.53 \pm 0.227$  mm), from onshore mating pairs (López-Cortegano et al., 2020). In the present study, the Gaussian

Single				Male 1			
0.1	0.6	0.6	0.6	0.2	0.1	0.1	0.1
0.1	1	0.6	0.6	0.6	1.6	0.6	0.6
0.1	0.6	1	0.6	0.6	0.6	1.6	0.6
0.1	0.6	0.6	1	0.6	0.6	0.6	1.6
Male 2				Multiple			
0	0	0	0	0	0.6	0.6	0.6
0.6	1.2	0.6	0.6	0	1.8	0.6	0.6
0.6	0.6	1.2	0.6	0.6	5.3	5.3	0.6
0.6	0.6	0.6	1.2	0.6	0.6	8.8	7.6
Wild							
0	0.6	0.6	0.6				
1	1	0.6	0.6				
0.6	1.3	1.6	0.6				
0.6	0.6	3	6.5				

**Figure 4.** Simplified mating models, where the choice value (C) is presented relative to the value of the C<sub>22</sub> cell from the single-choice design (C = 1.8; to recover the original values from Table 1, multiply the cells by 1.8). The choice parameters (C and B) in each model are in red. Male size classes in columns and female ones in rows.

mating preference function captured the essence of the mate choice process in this species, by identifying an excess of expected matings between similarly sized males and females as well as by estimating the bias in the laboratory experiments (multiple choice = 1.4 mm, i.e. males preferred females 1.4 mm bigger than themselves on average) and from the onshore observations (wild data = 0.74 mm, which falls within the 95% confidence interval of the previous estimate from López-Cortegano et al., 2020).

The mate choice mechanism is, however, more complex than presumed under the Gaussian mating preference function and the current analysis of the different mating models allows a fine-scale dissection of the mechanisms behind the choice of mates in this snail. In general, all designs showed a basic similarity-based mate choice mechanism, as mates of similar sizes were generally favoured (>1  $C$  parameters along the diagonal, Fig. 4), although the mechanism becomes more complex in multiple-choice and wild designs.

The single-choice design is the simplest design and is assumed not to incorporate potential mechanisms such as male or female intrasexual competition, as well as to have diminished strength of mate choice as male–female interactions among different sizes are not available (Dougherty & Shuker, 2015). The mating model that best fits this design showed a positive tendency for mating to occur among similar sizes, except for males of the smallest size class. It appeared that these small males are less likely to mate. This may be the result of a proportion of the smallest male size class being functionally immature, although previous studies have suggested that snails of this species should be sexually mature at > 3 mm (based on visual observation of developed sex organs and not on mating activity; Mak, 1996), or because they are more sensitive to the experimental manipulation. On this basis, if this size class is removed from the analysis, the interpretation is simple, with a perfect similarity-like mechanism and a tendency of mating with similarly sized individuals nearly twice as frequently as other sizes.

A relatively simple mating model was estimated from both the male choice designs when several female types were presented to every male. The trend is similar to the single-choice design, but in this case the reduction in mating efficiency occurs in the smallest female size class (i.e. males avoided to mate with the smallest females). Similar to the smallest males, it is likely that a portion of the smallest females are sexually inactive. When the smallest size class is excluded, again, a perfect similarity-like mechanism emerges. The opportunity for a male to choose among different female types therefore increases the strength of the mate choice mechanism. Mate choice for size has also been shown in the river bullhead fish, *Cottus gobio*, with the female being the sex making the mate choice (Bisazza & Marconato, 1988, using a female choice design).

Interestingly, the multiple design and onshore natural population observations showed nearly perfect fits to the same mating model, which further supports the underlying biological mechanism behind mate choice in this species. If we ignore the results of the smallest size classes (as discussed above), we have an interesting, two-component mating model, with a similarity-based mate choice mechanism ( $C$  parameter > 1 along the diagonal; Table 2) but including also a bias ( $B$  parameter > 1; Table 2), as would be expected from experimental estimates using quantitative approaches (López-Cortegano et al., 2020; Ng et al., 2019b), although different choice and bias were estimated for different size classes (i.e.  $C_2 \neq C_3 \neq C_4$ ;  $B_2 \neq B_3$ ). Again, excluding the smallest size classes, the picture is simplified with a good approximation to a Gaussian mating preference function plus certain bias, but showing deviations for the largest size classes in natural populations. The Gaussian mating preference function proposed earlier (based on  $C$  and  $B$  parameters; López-Cortegano et al., 2020; Ng et al., 2019b) can, therefore, be considered a reasonable approximation to mate choice in this species.

The comparison of different mate choice designs, however, reveals extra information. Single-choice and male choice designs did not differ qualitatively except for the smallest size classes, which is likely to be an artefact caused by immature specimens in these classes. The strength of mate choice was, however, increased by 50% under male choice ( $C \approx 3$ ) as compared to single-choice designs ( $C \approx 2$ ; Table 2), which suggests that having a variety of female sizes to choose from increases the likelihood of males choosing a certain size of females.

To distinguish the relative importance (directly being present or indirectly via a mucus trail) of the presence of other males, we included the second version of the male choice design (Male 2), where other males were allowed to move within the mating chamber, laying mucus trails for 1 h and then removed just before the experiment began. This alternative male design, however, presented the same trend of the classic male choice design (Male 1), suggesting that the physical presence (not only the mucus trails) of other males is necessary to alter the mating behaviour of the males, although the mechanisms (e.g. pheromone release, movement) and reasons (e.g. minimizing competition) for such behavioural change are unclear. It is possible that males exhibit behaviours that influence other males, but we did not observe any noticeable male–male interactions during the experiments.

Single-choice and male choice designs did, however, differ considerably even qualitatively from the multiple-choice design and onshore observations. The presence of other males, therefore, does seem to change the mating behaviour of males, increasing the strength of mate choice in the laboratory. Interestingly this is not a result of males fighting for mates (as has been recorded in natural populations, Ng, Davies, et al., 2016), as this was rarely observed in the experimental trials (personal observation), which suggests that a male, in the presence of other males, simply changes its mating strategy. There are no records of any sexual (either water or aerial diffused) pheromones in this snail; however, both water and aerial pheromones have been shown to contribute to sexual behaviour and affect partner 'attractiveness' in a closely related species, *Littorina littorea* (Erlandsson & Kostylev, 1995; Seuront & Spilmont, 2015), and so this remains a possibility. It is known that males do preferentially follow certain female sizes when searching for a partner via mucus trail following (Ng et al., 2019b), but this process does not seem to be applied to male trails as similar results were found in Male 1 and Male 2 designs. Another possibility is that the presence of several males might help to stimulate the sexual predisposition of females, which is a hypothesis that could be tested in the future.

The comparison between multiple-choice design and observations from the natural population provides further insights. The multiple-choice design detected the highest strength of mate choice compared to other designs (as indicated in Dougherty & Shuker, 2015) and even higher than in the onshore data ( $C_2$ ,  $C_3$ ,  $B_2$  and  $B_3$  were significantly higher in the multiple-choice design than the onshore data). However, when excluding the smallest size classes, the mating models are rather similar, suggesting that these designs only differ in the general strength of the choice and the parameters for some of the size classes. We interpret these differences as a consequence of two possibilities. First, this could be caused by the combination of certain experimental artefacts. In the multiple-choice experiments, contiguous classes differed by 1–3 mm, while the natural population data did not include these gaps among size classes, which could lead to a higher expected mate choice estimate in the laboratory study. Second, the natural population data showed a normal distribution of sizes (so intermediate size classes are more frequent), whereas each of the multiple-choice experiments had a male and a female of each size class (i.e. a uniform distribution). These two phenomena combined



could explain at least part of the difference between parameters estimated for the multiple-choice design and natural population data. Finally, despite the close resemblance in terms of the available interactions and choices of mates between the multiple-choice design and onshore observations, the two environments are fundamentally different; in the mating chamber, the eight snails (four males and four females) were placed in a simple, spherical environment with no cue of directionality except gravity, while in the natural environment, the direction of movement of these snails is primarily determined by the tides (possibly in response to tidal cues), as they perform tidal shuttling (i.e. moving up and down with the rising and ebbing tides; Li, 2012). This movement behaviour, as well as foraging that occurs concomitantly, may influence the mating behaviours and thus result in different choice and bias parameters estimated from the multiple-choice experiments and the onshore mating data. In the mating chamber, assuming that pheromones (or any other mechanism) play any role, the snails would be constrained to a relatively small space and this influence may, potentially be more effective. Nevertheless, although the cause of the difference requires further investigation, it is reasonable to assume that the choice estimates obtained from laboratory experiments represent the maximum choice for defined size classes, while the estimates obtained from onshore data represent a realistic estimation in the natural environment.

While we found strong evidence supporting the hypothesis that the mechanism of mate choice for size is based on a similarity-based preference plus a bias for larger size, few studies have proposed a similar mechanism. Bisazza and Marconato (1988), for example, demonstrated size-assortative mating in the river bullhead fish and found that females (which is the choosy sex; average length approximately 11 cm) preferred to mate with males 1.5 cm longer than themselves, but the preference was reduced for males that were either smaller than the female or larger than this size difference. The bias detected in this fish was a bit smaller than for *E. malaccana* in the multiple-choice design (approximately 1.4 mm in 8 mm of male size on average). Bertorelle, Bisazza, and Marconato (1997) studied the same fish and compared eight alternative mating models (including different female and male tactics to move and search for partners) using a computer simulation to study the comparative best fit to the observed data and adopted one particular model in which the preference is maximized after passing a certain size threshold. The degree of sophisticated behaviour (including alternative movement tactics) simulated for the fish are greater than would be expected for intertidal snails. These authors did not, however, use any similarity-based models in their study and so the potential application of any Gaussian-like mating preference function requires further investigation. A study on red-sided garter snakes, *Thamnophis sirtalis*, also showed that positive size-assortative mating is generated because male snakes prefer to mate with females of similar size (Luo et al., 2014), in a similar fashion to a Gaussian mating preference function. Other organisms have, however, been shown to have apparently different mechanisms than the one detected in our study. Perhaps the most common hypothesis is that positive size-assortative mating arises because both sexes prefer to mate with the biggest partner (Andersson, 1994; Crespie, 1989), which has gained direct support in some organisms (for example in the Japanese medaka fish, *Oryzias latipes*; Howard, Martens, Innis, Drnevich, & Hale, 1998; coral reef cardinal fish, *Sphaeramia nematoptera*; Rueger, Gardiner, & Jonesa, 2016). The present similarity-based mechanism and the one based on preference for the largest individuals are, however, easily confounded if the experimental design is focused on establishing just the preference for the largest (a bias) size class. To distinguish alternative mechanisms, approaches such as the model selection used in the present study or those used by

Bertorelle et al. (1997) are necessary to compare multiple models and select the best fit to the observed data.

### An Evolutionary Perspective

In this study, we disentangled the biological mechanisms that drive mate choice for size in the rocky shore snail, *E. malaccana*. The process is more complex than expected, although it resembles the similarity-based mechanism previously described including choice *C* and choice bias *B* parameters (e.g. López-Cortegano et al., 2020; Ng et al., 2019b). The strength of mate choice, however, changed at certain size classes, decreasing at the smallest size class and increasing at the largest size classes (at least from individuals observed on the shore). The possible reasons for the reduction in the smallest size class have already been discussed (most small snails may be immature or at least less sexually active) but the reason for the increase in the largest size class is less obvious. One possibility, as in other species like terrestrial salamanders and land snails, would be that choosing the largest females may have a clear advantage as they have the highest fecundity (Eddy et al., 2016; Yu & Wang, 2013) or even due to other reasons when it is the female that is the choosy sex (Luo et al., 2014). There does not, however, seem to be any relationship between size and fecundity in *E. malaccana* (Lau et al., 2017; Mak, 1998). One possibility that could explain this increased mate choice strength in the largest size class in natural populations could be that these (i.e. large) snails may win all mating fights (against other size classes, see Ng et al., 2019b) and, therefore, male competition would contribute positively to the corresponding parameters. Finally, this could be simply a problem of the population size distribution itself, as the largest males available are at the limit of the species' size distribution. These large males may choose to find females bigger than themselves, but as they are rare, they choose the largest size they can locate, which is a similar size class, resulting in an increase in matings for this combination ( $C_4 \gg C_3$ ; Table 2, Fig. 4). This issue would not, however, arise in the multiple-choice designs in the laboratory because, in this environment, the same number of female sizes were available.

Nevertheless, from an evolutionary perspective, these findings lead to two related new questions. First, is this mechanism frequent or similar in different species and taxa? It is already known that at least positive assortative mating is common (Jiang et al., 2013), and so, where possible, the mechanism driving these mating patterns in other species needs to be established experimentally. Second, how did this mechanism evolve? Or, in other words, which life history or ecological conditions may coincide in order to evolve such a potentially common mate choice mechanism? Since at least positive assortative mating is common in many species, it is possible that this combination of conditions may also be common in these species. To answer these questions will, however, require further theoretical and empirical investigations.

### Conclusions

Mate choice for size has been studied using an integrated approach of different laboratory experimental designs and onshore observational data. This approach has revealed several new findings to add to the general understanding of mate choice: first, the same similarity-based mechanism was confirmed, at least as a reasonable approximation, which included a choice and a bias parameter; second, as both sexes meet at high density in the natural populations, the multiple-choice design seems the most realistic representation of the natural scenario and the results were the most similar to the mating patterns in the natural populations (and even detected a higher strength of mate choice). Finally, these findings help identify future directions to test for the generality of

these parameters in other species and to characterize the physiological/genetic mechanisms responsible for them. The characterization of the biological mechanism behind mate choice in this species, therefore, allows a new perspective to improve our understanding of this key evolutionary trait.

### Author Contributions

A.C.R., G.A.W. and E.R.A. designed the study. S.L.Y.L. and E.R.A. conducted the behavioural experiments. A.C.R. carried out the analyses on mating models and E.R.A. the rest of the analyses. All authors contributed to writing the manuscript and gave final approval for submission.

### Data Availability

The experimental raw laboratory data are available in [Supplementary Table S1](#), while the summarized multiple-choice data in classes is available in [Supplementary Table S2](#). The *E. malaccana* original onshore data are available from DRYAD (<https://doi.org/10.5061/dryad.h214h8t>).

### Declaration of Interest

We declare we have no competing interests.

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### Supplementary Material

Supplementary material associated with this article can be found online at <https://doi.org/10.1016/j.anbehav.2021.02.020>.

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