Title

How pre- and postcopulatory sexual selection influence male mating decisions in a promiscuous species

When females mate multiply, male reproductive success depends on both pre- and postcopulatory processes, including female choice and sperm competition. However, these processes can favour different mating tactics in males. Here we use the Trinidadian guppy (*Poecilia reticulata*) system to understand how this conflict is resolved. We ask whether knowledge of recent female mating history leads males to adjust their mating effort in respect of the time devoted to mating activity, and the frequency and the sequence of mating tactics employed. To do this we quantified male mating behaviour in three competitive scenarios: 1) Single, when a focal male arrives near a single female and remains alone with her; 2) First, when a focal male is joined by a rival male; and 3) Second, when a focal male arrives after a rival male. We hypothesize that males adjust their behaviour based on arrival order. If female sequential mate choice is the main process shaping male mating behaviours (favouring First males in guppies), males should avoid competition and invest most when Single. Alternatively, if last-male sperm precedence is the major driver of decision making, males should invest more in mating attempts in the Second scenario. Greatest investment when First implies an intermediate strategy. We find that order of arrival influences mating decisions with most mating activity during the First scenario instead of the Single and Second scenarios. This result suggests that both pre- and postcopulatory processes influence mating investment, and that individual males make contingent decisions to maximize both mating and fertilization success.

Keywords:

Courtship display; female sequential mate choice; male-male competition; polyandry; sneak; sperm competition; unsolicited attempts.

When females mate with multiple males within the same breeding season, often referred as polyandry, males gain more mating opportunities but face, at the same time, the challenge of cryptic female choice (Eberhard, 1996) and sperm competition (Parker, 1970; 1998). This means that both precopulatory and postcopulatory processes influence the evolution of male sexual traits.

There is considerable interest in the contribution of secondary sexual traits to male reproductive success both during and after mating, and how they are influenced by pre- versus postcopulatory processes (reviewed by Evans & Garcia-Gonzalez, 2016). However, the relative influence of these two selective forces on male mating sexual traits continues to be debated (Buzatto, Roberts, & Simmons, 2015; Collet, Richardson, Worley, & Pizzari, 2012; Devigili, Evans, Di Nisio, & Pilastro, 2015; Pélissié, Jarne, Sarda, & David, 2014; Pischedda & Rice, 2012; Turnell & Shaw, 2015). Recent studies have focused on physical and sperm traits (e.g. body and sperm length, respectively; Evans & Garcia-Gonzalez, 2016), but few have considered mating behaviours (Buzatto et al., 2015; Devigili et al., 2015; Turnell & Shaw, 2015).

As with other male sexual traits, mating behaviours are subject to both pre- and postcopulatory sexual selection pressures (Andersson & Simmons, 2006), and are correlated with male reproductive success (Buzatto et al., 2015; Devigili et al., 2015; Fisher, Rodríguez-Muñoz, & Tregenza, 2016; Pélissié et al., 2014; Turnell & Shaw, 2015). Unlike most physical traits however, behaviours can be adjusted in light of the social context. For instance, mating behaviours can both promote mating and fertilization success by stimulating or circumventing female mate choice (e.g. courtship displays and unsolicited mating attempts, respectively; Gross, 1984; Andersson, 1994), and by avoiding or overcoming mating competition (e.g. mate guarding and sneak, respectively; Andersson, 1994; Neff & Svensson, 2013).

Male order of arrival at or near a female can greatly affect male reproductive success, both at the pre- and postcopulatory levels (Evans & Garcia-Gonzalez, 2016; Pélissié et al., 2014; Pischedda & Rice, 2012). For instance, when females choose sequentially (Jennions & Petrie, 1997; Real, 1990) a male's mating success will vary if he is the first or last to arrive near a female, depending on whether females are less or more discriminating (choosy) towards a first than a second male. Similarly, when sperm competition occurs, male fertilization success can only be maximized if he mates first or last, depending on whether there is first- or last-male sperm precedence (Birkhead & Hunter, 1990; Wedell, Gage, & Parker, 2002; Dosen and & Montgomerie 2004; Plath & Bierbach, 2011). Female mating history can thus play a crucial role in determining which mating behaviours a male should adopt.

In natural conditions males may have little opportunity to evaluate female mating history (Parker, Ball, Stockley, & Gage, 1997), raising the question of how males cope with this uncertainty. Considering this, we hypothesize that, if mating order has no effect on male mating decisions, a male should approach and invest in mating attempts whenever near a female. In contrast, a male could adjust his behaviour based on whether he arrives before or after a rival male.

Males face a particularly challenging decision when pre- and postcopulatory processes favour different mating orders. This arises, for example, when females are less choosy towards the first male they encounter, but where sperm precedence favours a male that mates afterwards. According to the trade-up hypothesis, females benefit from being less choosy with a first male, particularly when males are scarce, because they can ensure the fertilization of all their eggs (Halliday, 1983; Jennions & Petrie, 2000). Females may then become progressively choosier, and mate with any higher-quality males they subsequently encounter to enhance the genetic quality of their brood.

This hypothesis has been supported in species of birds (e.g. Gabor & Halliday, 1997), mammals (e.g. Klemme, Eccard, & Ylönen, 2006), insects (e.g. Bateman, Gilson, & Ferguson, 2001), fishes (e.g. guppies; Pitcher, Neff, Rodd, & Rowe, 2003), and reptiles (e.g. Laloi, Eizaguirre, Fédérici, & Massot, 2011).

Here, we ask whether males adjust their mating decisions over a short time scale based on the order at which they encounter a female. We further examine whether female mate choice or sperm precedence have the strongest influence on this decision making. To answer these questions, we studied the mating behaviours of male Trinidadian guppies (*Poecilia reticulata*) in mixed-sex groups. In these tests we mimicked the situation in the wild by allowing free interactions between individuals. First, we determined if a focal male approaches a female before or after another male (approach decision), and, second, whether his investment in mating behaviours depends on order of arrival (behavioural adjustment; Figure 1). The Trinidadian guppy is a freshwater livebearing fish. In this species, the precopulatory process of female mate choice should favour the first male to approach since females are less discriminatory towards him than towards subsequent males (Houde, 1997; Liley, 1966; Pitcher et al., 2003). However, sperm competition (postcopulatory process) favours the last male to mate since mixed paternity broods (Becher & Magurran, 2004) are predominantly sired by these males (Evans & Magurran, 2001; Pitcher et al., 2003).

Male guppies perform two mating tactics: consensual courtship displays and unsolicited mating attempts (Magurran, 2005). Courtship displays result in the greatest paternity success (Evans & Magurran, 2001). Unsolicited mating attempts, on the other hand, do not require female cooperation (Houde, 1988; Magurran, 2005) and typically result in the transfer of only modest amounts of sperm (Pilastro & Bisazza, 1999). This mating tactic is more frequent when other males are present (Magellan, Pettersson, & Magurran, 2005; Magurran, 2005).

We hypothesize that, if males are able to evaluate female mating history based on their own assessment of male-male competition, they will adjust their mating behaviour based on whether they are with a female alone (Single), approached before (First) or after (Second) a rival male (Figure 1). In more detail, if female mate choice gives the most advantage to males, they should avoid competition during mating, and invest more in following and trying to mate when they are alone with a female (Single), particularly using the mating tactic that allows them to transfer more sperm (courtship display). In this case, we expect male guppies to approach females with no rival male following them, to court more and repeatedly, and to spend more time with a female when Single. On the other hand, if sperm precedence gives the most advantage to males, they should invest more when there is competition, particularly in the mating tactic that transfers fewer amounts of sperm, but may help secure last sperm precedence (unsolicited attempt). In this case, we predict that male guppies should approach females with at least one rival male following them, perform more unsolicited mating attempts, and spend more time with a female when Second. However, if both pre- and postcopulatory processes are important, males should invest more when First, again due to potential advantages in terms of female choice, and, simultaneously, to secure sperm precedence. Figure 1 summarises these scenarios.

Alternatively, if information available to males during the current encounter conveys little fitness benefits to them, then order of arrival should not influence their behaviour towards the female. Here we expect no difference in mating behaviour if a male is the only, the first or the second to approach a female (Figure 1).

Materials and Methods

Experimental setup

We used descendants from wild guppies from the Lower Tacarigua River, in Trinidad. Following other studies with guppies (e.g. Deacon, Ramnarine, & Magurran, 2011), observations were carried out in two mesocosm tanks (100 cm x 56 cm x 30 cm). Behavioural observations in mesocosms have the advantage of allowing individuals to behave and interact more freely (Devigili et al., 2015). Each mesocosm contained gravel, an aerating system and two thermostat heaters. The range of temperatures (24.1ºC to 25.7ºC) was similar to that found in the wild (Reeve et al., 2014).

Inside each mesocosm we placed one mixed sex group of fish: four males and three females. Wild females are only receptive either as virgins or in few days immediately following parturition (Liley, 1966; Liley & Wishlow, 1974), thus male guppies are expected to typically encounter non-receptive females in the wild. This way, to better simulate natural situations, all females used in our experiments came from a stock tank, thus likely to be non-virgin and non-receptive.

Two groups of three females were haphazardly chosen (standard length mean ± SE: 2.09mm ± 0.45) from the same stock tank and allocated to each of the two mesocosms in the afternoon on the day before the observations. This allowed females to acclimate to the new conditions. Simultaneously, 16 males were transferred from stock tanks to four maintenance tanks (30 cm x 15 cm x 20 cm). Males were kept in all-male groups to ensure they were not sperm limited and, therefore, were sexually active during the observation period. On the day of the observations, males were haphazardly chosen (standard length mean ± SE: 1.48mm ± 0.15) and assigned to one of the mesocosms 15 minutes before observations started. To avoid familiarity during observations, males came from different stock and maintenance tanks from that of other males and females.

During each observation day, we tested two male groups in each mesocosm with the same female group, meaning each female group was used twice.

All observations were conducted between 9 am and 1 pm. All females were fed with flake food one hour before the beginning of the observation day, while males were fed on the previous evening to increase mating behaviour frequency (Sartori & Ojanguren, n.d.). In total, we tested 152 males and 60 females. At the end of an observation day, all tested individuals were transferred to a tank identified as observed group to ensure that males were tested only once, and females were only tested in one day (with two male groups).

Experimental design

The behaviours of the four males in the mesocosm were observed one at a time and registered using JWatcher v1.0. (Blumstein & Daniel, 2007). To ensure each male was sampled only once, prior to observation each male was identified based on their unique colour patterns (Magurran, 2005). Each male behaviour was registered while following a female, starting with the approach to her and ending when he left that female. As a result, the duration of each observation was neither controlled by the observer nor had the same length for each focal male. Instead, the duration of each observation was determined by the focal male's decision to swim away from the female. Each focal male experienced one of three possible competitive scenarios: Single, First and Second (Figure 1). Single was defined as when a male approached a solitary female and remained as the only male following her the entire time. First was defined as a situation when the male approached a solitary female, but was subsequently joined by at least one rival male. Second was defined as a situation where the male approached a female that was already being followed by at least one rival male. Therefore, we conducted a quasi-experimental design (Cook, 2015) in the sense that males were not allocated to a given scenario, but scenarios emerged from males (focal and rivals) decisions.

Behavioural recording

Males made a sequence of mating decisions: (1) whether to approach a female alone or already being followed by one or more males (approach decision), and (2) whether and how to adjust their mating behaviour while following the female (behavioural adjustment) As behavioural adjustment we considered: (a) time spent following the females, (b) mating tactics performed (courtship displays or unsolicited mating attempts), and (c) sequence of mating behaviours.

We employed two sampling methods: focal and scan sampling. Focal sampling was used to record male mating decisions and classify the pertaining competitive scenario. During focal sampling we recorded the total time each focal male followed a female, the frequency of mating tactics performed (courtship displays or unsolicited attempts), and the sequence of mating behaviours (from the time the male approached a female until he swam away from her). Scan sampling was used to calculate the probability that males had of finding females swimming alone (non-competitive encounter) or with a rival male (competitive encounter). This method allowed us to have a proxy of competition. Each focal sampling started when the focal male approached a female and ended when he left the female. Scan sampling was performed between focal samplings.

We characterized the prevailing competitive scenario during each focal sample by recording the presence of any other male near the female, as well as the order of arrival of the focal male. Changes in the competitive scenario during the time a focal male followed the female were not considered. For example, we considered a scenario as First even if the rival male swam away while the focal male was still following the female.

We started each trial with a scan sample and alternated between scans and focal observations. Each trial consisted of five scan samples of the females and four focal observations, one assigned to each male at a time. A total of 184 scans and 152 focal samples were performed.

Statistical analyses

All analyses were performed using the software R 3.2.3 (R Core Team, 2015); the significance alpha was set at $P = 0.05$.

Approach decision

To evaluate if males preferred non-competitive versus competitive encounters, relative to a random expectation, we first estimated the frequency of each encounter during the scan samples. In other words, we estimated the probability of females being alone or with at least one male following them. These probabilities were calculated by dividing the frequency of scan samples with females swimming alone, and with females with at least one male following them, by the total number of scan samples, respectively. A chi-square goodness-of-fit test was then used to evaluate males' approach decision against the null expectation.

Behavioural adjustment while following a female

Time following

To test if total time following a female was dependent on the competitive scenarios, we ran a linear mixed-effects model (LME) from the package nlme (Pinheiro, Bates, DebRoy, & Sarkar, 2014). The model included time following as the response variable, the competitive scenario (Single, First, or Second) as a fixed term, and the mesocosm and the female group as random terms, with the female group nested inside the mesocosm. Diagnostic plots revealed departure in residuals homogeneity in the response variable. Therefore, time following the female was log-transformed. Models were compared with a LM model with no random terms using a likelihood ratio test (LRT).

Additionally, we ran an unpaired t-test statistic to compare the time that First males spent with a female before the approach of another male with the total time following a female by Single males. This allowed us to infer if time following a female when in the First scenario was dependent on the male's order of arrival alone, and not on the time that he previously invested with that female.

Mating tactics frequency

We considered the three competitive scenarios to analyse the effect of other males' presence, and order of arrival, on the focal male's frequency of courtship displays and unsolicited mating attempts. For the First scenario, only the behaviours performed after the approach of a rival male were considered. To account for the excess of zeros, the frequency of mating behaviours was analysed considering separately zeros and non-zeros using a hurdle model (package pscl; Zuur, Ieno, Walker, Saveliev, & Smith, 2009). This procedure considers a binomial distribution with a logit link function to model the zeros and a Poisson or a negative binomial distribution to model the non-zeros. Response variables were the frequency of mating tactics (courtship displays and unsolicited attempts, analysed separately), while competitive scenario and mesocosm were added as explanatory variables, both for the count (non-zeros) model and for the zero model. A likelihood ratio test of nested models with a backward step-wise procedure was used to select the best-fit model (LRTEST from the package lmtest; Zuur et al., 2009).

Mating behaviours sequence

Our aim was to examine if the probability of each behaviour was dependent on the behaviour that preceded it, and on the competitive scenario the focal male found himself in. As before, when the focal male was the first to approach a female, we only considered the behaviours performed after the approach of a rival male. We evaluated sequences of two behaviours (one followed immediately by another). There were three possible precedent behaviours: approach, courtship display and unsolicited attempt. Each of these behaviours was analysed separately. In all cases, a sequence terminated with either a courtship display, or an unsolicited mating attempt, or a swim away. Hence, for each analysis three sequence types were considered: precedent behaviour-courtship display, precedent behaviour-unsolicited attempt, and precedent behaviour-swim away.

Our data do not fulfil the assumptions of Markov Chain Analysis, since the transition times between behaviours were not homogeneous, and the probability of behaviours was not uniform (Bakeman & Gottman, 1997). We, therefore, applied generalized linear mixed-effects models (GLMER from the package lme4; Bates, Mächler, Bolker, & Walker, 2014), with a logit link function for a binomial distribution to each precedent behaviour. The response variable was the occurrence/non-occurrence of each sequence type. This means that in the data set, each focal male was represented three times, one time for each of the three sequences, using “1” to indicate the occurrence of one sequence and “0” the non-occurrence. Fixed terms considered were the competitive scenario and sequence type, as well as their interaction. Random terms were female group nested in the mesocosm, as well as the repeated observations of each focal male (the pseudo-replicates). Model selection was based on a backward step-wise procedure with an analysis of variance (anova).

Ethical notes

Before observations all individuals were kept in mixed-sex stock tanks in, with similar densities. The stock tanks had gravel in the bottom, and an aerating system. The room was kept at a controlled stable temperature. The period of light was controlled (12:12h light:dark cycle). Individuals were fed daily. Before the observations, all individuals were transferred to observation tanks (mesocosms) to acclimate to the new conditions. Female guppies are often more sensitive to changes than males, because of their stronger and more stable social bonds (Croft et al., 2006; Griffiths & Magurran, 1998). Therefore, females were transferred to the mesocosms several hours before the observations and were kept with other familiar females, while the males were transferred 15 minutes before observations started. After observations all individuals were transferred to stock tanks with no fish (labelled as “observed individuals” during the experiment). All animals were individually and carefully caught and moved between tanks with a net.

All behavioural observations were carried out at the School of Biology at the University of St Andrews. The premises where the observations were carried out comply with the UK guidelines for the treatment of animals in behavioural research and teaching, set by the UK Home Office (PCD 60/2609). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Results

Approach decision

In 45.7 % of the scan samples (out of a total of 184) females were not followed by any male. However, 59.2 % of focal males approached females when they were alone. There was no significant difference between the frequency with which males chose or avoided competition relative to the null expectation ($\chi^2_1 = 1.5$, $N = 152$, $P = 0.220$). After approaching the female, the focal male remained the Single one following the female in 29.6 % of the cases; was the First to approach but other male approached afterwards 29.6 % of the time; and was the Second to approach in 40.8 % of the cases.

Behavioural adjustment while following a female

Time following

For the time males spent following females, only the competitive scenario remained in the best explanatory model ($F_{2,149} = 16.2$, $N = 152$, $P < 0.001$). On average, males spent 25 seconds following a female. No significant difference was found between the time spent following a female when males were Single versus when they were Second ($|t| = 0.6$, $P = 0.568$). However, males that First approached a female spent more time following her compared to males that were Single or the Second to arrive at the female (Single vs. First: $|t| = 5.1$, $P < 0.001$; First vs. Second: $|t| = 4.9$, $P < 0.001$; Figure 2). Additionally, the total time males spent following the female when they were First was independent of the time invested prior to the approach of another male. The time that First males spent with a female before the approach of another male was not different from the total time following when males were in the Single scenario (unpaired t-test: $|t|_{195} = 0.6$, $N_1 = 90$, $N_2 = 107$, $P = 0.555$).

Mating tactics frequency

Most focal males did not perform any courtship display (86.2 %) or unsolicited attempt (78.9 %) when following females. From those that performed mating attempts, they did it only once in most of the cases: 81.0 % and 75.0 % of the samples for courtship displays and unsolicited attempts, respectively. Specifically, for the frequency of courtship displays, and after model selection, none of the explanatory variables (competitive scenario and mesocosm) was included in the best-fit model ($\chi^2_2 = 8.9$, $N = 152$, $P = 0.064$; Figure 3*a*). By contrast, the best-fit model that explained the frequency of unsolicited attempts included the competitive scenario ($\chi^2_2 = 8.2$, $N = 152$, $P = 0.017$; Figure 3*b*). Focal males that approached a female First performed more unsolicited attempts than males that were the Single following the female ($z = 2.3$, $P = 0.031$), or males that were the Second ($z = 2.3$, $P = 0.031$).

Mating behaviours sequence

For the analyses of the behavioural sequences that started with approach, the best-fit model ($\chi^2_4 = 10.2$, $N = 456$, $P = 0.037$) excluded the random terms and included the interaction between sequence type and competitive scenario. Based on the best model, males had higher probability of swimming away after an approach than of performing courtship displays or unsolicited attempts across all scenarios (Figure 4*a*, Table 1). However, this probability was higher when they were the Second to approach a female (see in Table 1, for comparison between Second and the other two scenarios).

Only 21 focal males performed at least one courtship display, meaning that only those males were included in the analysis of the behavioural sequences that started with a courtship display. Males never performed some sequences in some competitive scenarios (courtship display-courtship display when focal males were Single with the females, and courtship display-unsolicited attempt when males were the First to approach the females; Figure 4*b*). Differences between competitive scenarios were not significant, as the best-fit model included sequence type as the only explanatory variable ($\chi^2_2 = 21.9$, $N = 63$, $P < 0.001$). Males had significantly higher probability of swimming away after a courtship display, than of performing a second courtship ($z = 3.985$, $P < 0.001$) or an unsolicited attempt ($z = 4.283$, $P < 0.001$). But, when males did not swim away, they were equally likely of performing a courtship and an unsolicited attempt ($z = -0.867$, $P = 0.386$).

For the analyses of the behavioural sequences starting with unsolicited attempts, only the 32 focal males that performed at least one unsolicited attempt were considered. The best-fit model excluded the random terms (mesocosm and female group) and included the interaction between the competitive scenario and the sequence type ($\chi^2_4 = 11.0$, $N = 96$, $P = 0.026$). The best model revealed that the probability of swimming away after an unsolicited attempt was higher when males were Single and Second than when they were First (Figure 4*c*; Table 1). Additionally, there were no significant differences between the probabilities of First males swimming away, performing a courtship display or repeating an unsolicited attempt.

Discussion

Our findings indicate that males adjust their mating behaviour in response to the order in which they arrive at a female, investing more on mating behaviours when they precede a rival. Specifically, males spent more time following females and performed more and repeated mating attempts when they were the First to arrive at the mating context, than males that remained Single with the female or arrived Second. The scenario First was the situation where males' chance of being preferred by females (female sequential mate choice) and of fertilizing more eggs (sperm precedence) could be compromised by a rival's approach. By investing more in this scenario, males probably try to reduce such risks. Taken together our results demonstrate that males make contingent mating decisions depending on the competitive environment. However, they do this by investing more in unsolicited mating attempts than in courtship displays.

Approach decision

Males approached a female regardless of the presence or absence of other males, in line with the frequency of occurrence of competitive encounters in the population. This result indicates that order of arrival near a female does not influence a male's decision to approach a female.

Since male guppies express weak social bonds with females and are constantly moving between groups of females (Croft et al., 2006; Griffiths & Magurran, 1998), avoiding or seeking encounters with other males may be difficult in the wild. In fact, we found that, even in cases where a male approached a solitary female (choosing a non-competitive encounter), he was often joined by a rival male. It is likely that guppies do not avoid other males, because doing so does not prevent sperm competition, as it has been shown for the field cricket (*Gryllus campestris*; Fisher et al. 2016). At the same time, it seems unlikely that male guppies actively seek out females accompanied by rival males.

Behavioural adjustment while following a female

We predicted that if a male's order of arrival near a female was important in shaping male mating behaviour, we would find different patterns of mating behaviour across the three scenarios. More specifically, we expected more investment in mating behaviours when Single if males were prioritizing female mate choice, or more investment when Second if males were prioritizing sperm precedence. The First scenario was an intermediate one, where males would try to secure both the advantage of female choice and sperm precedence. We found evidence for the First scenario, with First males investing more in females than Single and Second males. However, they did so through unsolicited mating attempts instead of courtship display. Additionally, males postponed the decision to swim away, and invested more on mating attempts (courtship and unsolicited) after an unsolicited attempt when were First. This indicates that, despite seemingly being non-selective about their order of arrival, males do not invest equally in all three competitive scenarios, but invest more in the scenario where neither pre- nor postcopulatory processes dominate.

There is evidence that female guppies choose higher displaying males (Magurran, 2005) and that male reproductive success is linked to the frequency of courtship displays (Evans & Magurran, 2001). Since we found that focal males did not change the frequency of courtship displays between competitive scenarios, this could indicate that investment on courtship displays is more dependent on female traits, like receptivity (Farr, 1980; Guevara-Fiore, Stapley, & Watt, 2010), than on the male’s order of arrival. Males typically achieve mating after courtship display only if females are receptive (Magurran, 2005). Given that receptive females are more responsive to males that approach them first (Pitcher et al., 2003) and males are able to distinguish among receptive and non-receptive females (Guevara-Fiore, Stapley, Krause, Ramnarine, & Watt, 2010), a higher investment in courtship displays could still be expected with receptive females in scenarios where males were alone with the females or the first to approach. Although we did not control for female receptivity, our experimental approach mimics closely female-male dynamics in nature, where receptive females are rare (Liley, 1966; Liley & Wishlow, 1974). In such natural conditions, our results indicate that courtship displays are not used differently across the three competitive scenarios, suggesting that reproductive advantages are obtained from such displays irrespective of whether males are Single, First or Second with a female. Future studies could examine the relevance of female receptivity in male mating behaviours relative to order of arrival.

In contrast to courtship displays, both the frequency of unsolicited mating attempts and the sequence of behaviours performed after these attempts were dependent on the order of arrival. Males that were the First to approach the female performed more unsolicited attempts, and were more likely to repeat that behaviour or to court than to swim away than Single and Second males. It has been previously shown that male guppies increase unsolicited mating attempts in response to increased mate competition (Magellan et al., 2005; Magurran, 2005). Indeed, males can mate more rapidly and repeatedly without female cooperation by performing unsolicited attempts (Houde, 1988; Magurran, 2005). Furthermore, by re-mating, males ensure a higher chance of being the last one to mate or of transferring more sperm. The greater proportion of unsolicited mating attempts found in our study when males were the First to approach suggests that males are less willing to abandon the female in that scenario. This could represent a strategy either to transfer more sperm (correlated with unsolicited attempts frequency; Matthews, Evans, & Magurran, 1997), to be the last to transfer sperm and ensure last sperm precedence, or to inform rival males that the female has mated. Supporting this last hypothesis, a theoretical study suggests that males should only transfer such information to rival males when they are in disadvantage on sperm competition (Engqvist & Taborsky, 2017). In fact, the First scenario is the one where the risks are higher, as males have more to lose than those who approached Second or those that were alone with a female the entire time.

To assess if First males tried to be the last to mate, we performed additional statistical analyses on the order by which males left the females and found that males left the females later than rivals more frequently when they were the First to arrive than when they were Second (see Appendix Table A1). We also found that males invest more time and make more mating attempts when they left the female after the rival male in both First and Second scenarios (see Appendix Table A1, Figures A1 and A2), suggesting that First males tried to secure sperm precedence. These findings are compatible with higher investment in mating attempts (more sperm transference) in high sperm competition scenarios predicted by theoretical models (Parker et al., 1997), and supported by empirical studies in several other species (e.g. dominant versus sneaker males, Collet et al. 2012; and absence versus presence of competitor males, Kelly & Jennions 2011). Sperm competition is potentially high in guppies, as females can store sperm of multiple males for months (Herdman, Kelly, & Godin, 2004; Magurran, 2005) and a single brood is generally fathered by two or more males (Becher & Magurran, 2004; Devigili et al., 2015; Elgee, Ramnarine, & Pitcher, 2012). Males' investment in time and energy with a female, when they are the First to approach the female, could give them a significant fitness advantage relative to sperm competition. In fact, previous studies found that male guppies invest less in mating behaviours when they lose the opportunity of mating first with a female (Dosen & Montgomerie, 2004; Jeswiet, Lee-Jenkins, Ramnarine, & Godin, 2011).

Our study provides additional support for the finding by Magris and colleagues that, when female cryptic choice is controlled (by artificial insemination), there is first- instead of last-male sperm precedence (Magris, Cardozo, Santi, Devigili, & Pilastro, 2017). The greater mating investment by First males reported in our study may increase a male’s chances during cryptic female choice. Moreover, sperm competition could explain why First males performed more unsolicited attempts and repeated more mating attempts after this tactic. This was why sperm precedence was reversed in Magris et al. (2017) study where males’ equal ejaculate size competed for fertilization.

For female sequential mate choice, we predicted higher investment when males were Single, than when they were First and Second. We failed to detect an increase time following and in the frequency of courtship displays when males were Single (as discussed above). What we did find was that males spent more time with females when they were First and invested more in mating attempts (both unsolicited and courtship) after an unsolicited attempt than in the other scenarios. This suggests that female sequential mate choice is not the main mechanism shaping male mating behaviours, but that in concert with sperm precedence it selects for male mating tactics. We cannot exclude the possibility that males also improved their chances of being preferred by the females when arriving First. The fact that males invested in courtship displays after an unsolicited mating attempt supports this possibility, as do studies showing that female guppies cryptic choice favours the sperm of preferred males (Gasparini & Pilastro, 2011; Pilastro, Simonato, Bisazza, & Evans, 2004).

Conclusions

Our work advances understanding of how pre- and postcopulatory sexual processes shape male mating behaviours, when the order in which males approach a female (before or after a rival male) provides contrasting competition advantages. We provide, for the first time, empirical evidence about the interplay between male uncertainty about long-term female mating history, short term male behavioural flexibility, and antagonistic pre- and postcopulatory processes. We show that male guppies use information based on the order in which they approach a female and invest more in mating attempts when the risk of losing both female preference and sperm precedence is higher. In light of the growing evidence of female multiple mating across taxa (Arnqvist & Nilsson, 2000; Barbosa & Magurran, 2006; Parker & Birkhead, 2013; Taylor, Price, & Wedell, 2014), we suggest that future studies (including comparative approaches) ask how males (and females; Shuster, Briggs, & Dennis, 2013) shape their mating decisions based on their knowledge of their mates' mating history. Such new studies should consider the degree of polyandry in a population (based on the number of females that mate multiply, as well as the maximum average number of matings per female; Taylor et al. 2014); the degree of uncertainty about female mating history; whether sequential mate choice is in place or not; and the degree of sperm precedence (if partial or total; see Turnell & Shaw, 2015). For example, in species like guppies that have high frequency of female multiple mating, but unlike guppies have total sperm precedence, postcopulatory pressures may have a higher contribution to the evolution of male mating behaviours. In such cases, uncertainty about female mating history and order of arrival should be irrelevant, if males assume that they are always the first or the last to mate, and have strategies to control sperm competition (e.g. mate guarding; Ridley, 1980, 1989), or to avoid sperm competition (e.g. mating plugs; Dougherty, Simmons, & Shuker, 2016). On the other hand, precopulatory selection may act more strongly on male mating behaviours in species with partial sperm precedence and less polyandry than in guppies (Turnell & Shaw, 2015). Indeed, as we have shown here, the outcome of selection on male behaviour is subject to subtle drivers that vary in intriguing and complex ways amongst species.

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Tables

Table 1. Summary table of the GLMER models for the behavioural sequences on different competitive scenarios.

Precedent Behaviour	Sequence type			Competitive scenario	Z-value	P-value
Approach						
	Approach-Swim away	vs	Approach-Courtship display	Single	-5.013	**<0.001**
				First	-4.795	**<0.001**
				Second	-6.232	**<0.001**
				Single vs First	-0.057	0.955
				First vs Second	-2.015	**0.044**
				Single vs Second	-2.108	**0.035**
			Approach-Unsolicited attempt	Single	-4.429	**<0.001**
				First	-3.500	**<0.001**
				Second	-6.743	**<0.001**
				Single vs First	0.854	0.393
				First vs Second	-2.532	**0.011**
				Single vs Second	-1.620	0.105
Unsolicited attempt						
	Unsolicited attempt-Swim away	vs	Unsolicited attempt-Courtship display	Single	-2.948	**0.003**
				First	0.000	1.000
				Second	-1.736	0.083
				Single vs First	2.586	**0.010**
				First vs Second	-1.332	0.183
				Single vs Second	1.515	0.130
		vs	Unsolicited attempt-Unsolicited attempt	Single	-2.948	**0.003**
				First	-0.409	0.682
				Second	-2.128	**0.033**
				Single vs First	2.384	**0.017**
				First vs Second	-1.423	0.155
				Single vs Second	1.185	0.236
	Unsolicited attempt-Unsolicited attempt	vs	Unsolicited attempt-Courtship display	Single	0.000	1.000
				First	0.409	0.682
				Second	0.514	0.608
				Single vs First	0.198	0.843
				First vs Second	0.152	0.879
				Single vs Second	0.296	0.768

Analyses were conducted separately based on the behaviour that initiated the sequence type (precedent behaviour): approach to a female (*N* = 152; all males approached a female) or unsolicited attempt (*N* = 32; number of focal males that performed the unsolicited tactic). Each sequence type includes the precedent behaviour and the following behaviour (courtship display, unsolicited attempt, or swim away). Sequences that started with courtship display are not shown because the best-fit model did not include the interaction between sequence type and competitive scenario. P-values in bold represent significant differences.

Table A1. Summary table of the best models encountered to explain male mating investment relative to their chance of winning last sperm precedence.

Response variable	Model type		Distribution	Explanatory variables	Contrasts	Z-value/ T-value	P-value
Leaving order	GLMER		Binomial	Competitive scenario	First vs Second	-2.2	0.025
Time following	LMER		Normal (log-transformed)	Competitive scenario	First vs Second	-3.8	<0.001
				Leaving order	Earlier vs Later	4.8	<0.001
Courtship display	Hurdle	Zero model	Poisson	Leaving order	Earlier vs Later	5.0	<0.001
Unsolicited attempt	Hurdle	Count model	Negative Binomial	Competitive scenario	First vs Second	-2.2	0.0310
		Zero model		Leaving order	Earlier vs Later	5.0	<0.001

Two competitive scenarios during which focal males faced competition were considered (First and Second). Response variables were: leaving order (i.e., leave the female earlier/later than rival male), frequency of courtship displays, frequency of unsolicited attempts, and time following females. Explanatory variables considered for all the models were: competitive scenario (First and Second), mesocosm, female group (nested inside the mesocosm) and leaving order (except when it was used as the response variable). For leaving order (as the response variable) the best-fit model included the competitive scenario ($\chi^2_1 = 5.0$, $N = 107$, $P = 0.025$). Both leaving order and the competitive scenario were included in the best-fit model for time following ($F_{104,149} = 23.5$, $N = 107$, $P < 0.0001$; Figure A1). For frequency of courtship displays, only leaving order (as explanatory variable) influenced this behaviour (included in the zero model; $\chi^2_1 = 30.0$, $N = 107$, $P < 0.001$; Figure A2a). In relation to frequency of unsolicited attempts, the competitive scenario was included in the count model and leaving order was included in the zero model ($\chi^2_1 = 29.2$, $N = 107$, $P < 0.001$; Figure A2b). None of the best models included the interaction between leaving order and competitive scenario.

Figure legends

Figure 1. Questions, hypotheses and predictions on male mating decisions. Observed competitive scenarios: Single (*N* = 45), the focal male (black) was the single male following a female (grey) for the entire time; First (*N* = 45), the focal male was the first to approach the female and a rival male (white) arrived later; Second (*N* = 62), the focal male approached the female after the rival male. Males make mating decisions at different points: (1) whether to approach a female or not (approach decision), (2) after approaching, they decide how and how much to invest (time spent following, mating tactics and mating behaviours sequence). The scenario below each of the hypothesis represents the predicted outcome. In addition, the framework identifies the mating tactic we expect to dominate under each scenario. Drawings are adapted from Liley (1966).

Figure 2. Time spent by focal males following a female under the three competitive scenarios: when the focal male was the single male following a female the entire time (Single, *N* = 45); when he was the first to approach a female (First, *N* = 45); and when he was the second (Second, *N* = 62). Pairwise comparisons of time spent following between competitive scenarios were obtained from the best-fit LME model. Asterisks and associated lines above the plots show which sets of competitive scenarios differed significantly from one another (*$P < 0.05$). In each boxplot the internal line represents the median. Lower and upper edges represent the 25% and the 75% quantile, respectively. Whiskers below and above the box edges represent, respectively, the minimum and the maximum points within the 1.5 interquartile range. Circles represent outliers.

Figure 3. Frequency of mating tactics in relation to competitive scenario. Mating tactics were either (a) courtship displays (on the left side of the figure) or (b) unsolicited mating attempts (on the right side). There were three scenarios: when the focal male was the single male following a female the entire time (Single, $N = 45$); when he was the first to approach a female (First, $N = 45$); and when he was the second (Second, $N = 62$). The diameter of the circles is proportional to the sample size. Pairwise comparisons between competitive scenarios of the frequency of each mating tactic were obtained from the best-fit hurdle models. Asterisks and associated lines above the plots show which sets of competitive scenarios differed significantly from one another ($^*P < 0.05$).

Figure 4. Mating behaviours sequences in three different competitive scenarios. The three scenarios were (from left to right; with focal male represented in grey): when the focal male was the single male following a female the entire time (Single, $N = 45$); when he was the first to approach a female (First, $N = 45$); and when he was the second (Second, $N = 62$). Each arrow represents the sequence of two behaviours. Each sequence could start with (a) approach to a female, (b) courtship display, or (c) unsolicited attempt; and end with (b) courtship display, (c) unsolicited attempt, or (d) swim away. Arrow width represents the estimated probability of that sequence. Sequences that were not observed or were rare (with an estimated probability lower than 0.1) were not included. For each scenario, continuous arrows represent behavioural sequences that were significantly more likely to occur than sequences represented by dashed arrows. We obtained the probability estimates of each behaviour sequence from the best-fit statistical GLMER models. The diameter of the circles is proportional to the frequency of each behaviour for each scenario.

Figure A1. Time spent by focal males following a female between scenarios with different advantage in relation to sperm precedence. Two competitive scenarios related with order of arrival near a female were considered: when the focal was the first to approach a female (First); and when he was the second (Second). And, within these competitive scenarios, males were divided in two additional scenarios depending on the order they left the female (i.e. probability of mating at last): when the focal male leave the female earlier than the rival (lower probability of mating after the rival - Earlier); and when the focal leave the female later than the rival (higher probability of mating after the rival - Later). Pairwise comparisons of time spent following between scenarios were obtained from the best-fit LME model. Asterisks and associated lines above the plots show which sets of competitive scenarios differed significantly from one another (*$P < 0.05$). In each boxplot the internal line represents the median. Lower and upper edges represent the 25% and the 75% quantile, respectively. Whiskers below and above the box edges represent, respectively, the minimum and the maximum points within the 1.5 interquartile range. Circles represent outliers.

Figure A2. Frequency of mating tactics between scenarios with different advantage in relation to sperm precedence. Mating tactics were either (a) courtship displays (on the left side of the figure) or (b) unsolicited attempts (on the right side). Two competitive scenarios related with order of arrival near a female were considered: when the focal was the first to approach a female (First); and when he was the second (Second). And, within these competitive scenarios, males were divided depending on the order they left the female (i.e. probability of mating at last): when the focal male leave the female earlier than the rival (lower probability of mating after the rival - Earlier); and when the focal leave the female later than the rival (higher probability of mating after the rival - Later). The diameter of the circles is proportional to the sample sizes. Pairwise comparisons of the frequency of each mating tactic between scenarios were obtained from the best-fit hurdle models. Asterisks and associated lines above the plots show which sets of competitive scenarios differed significantly from one another (*$P < 0.05$).

***Acknowledgments**

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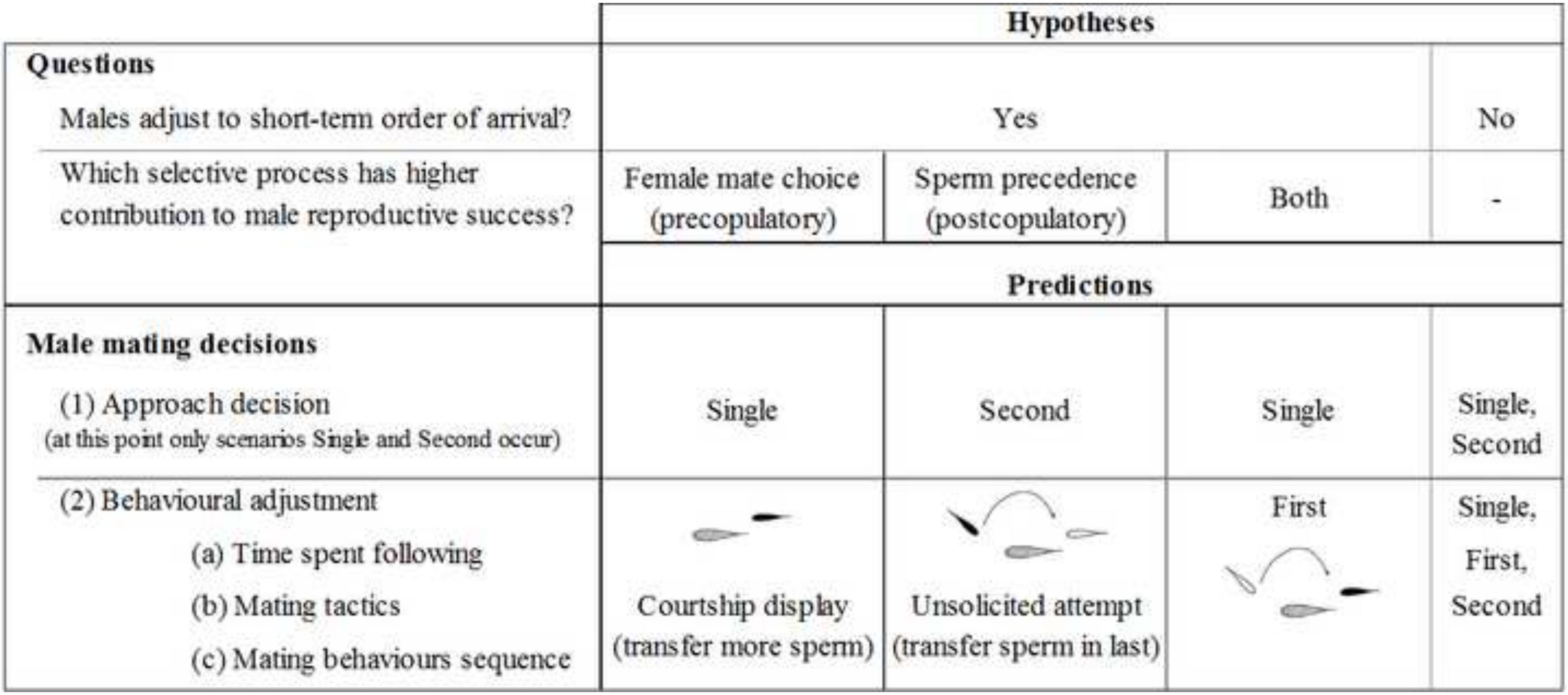
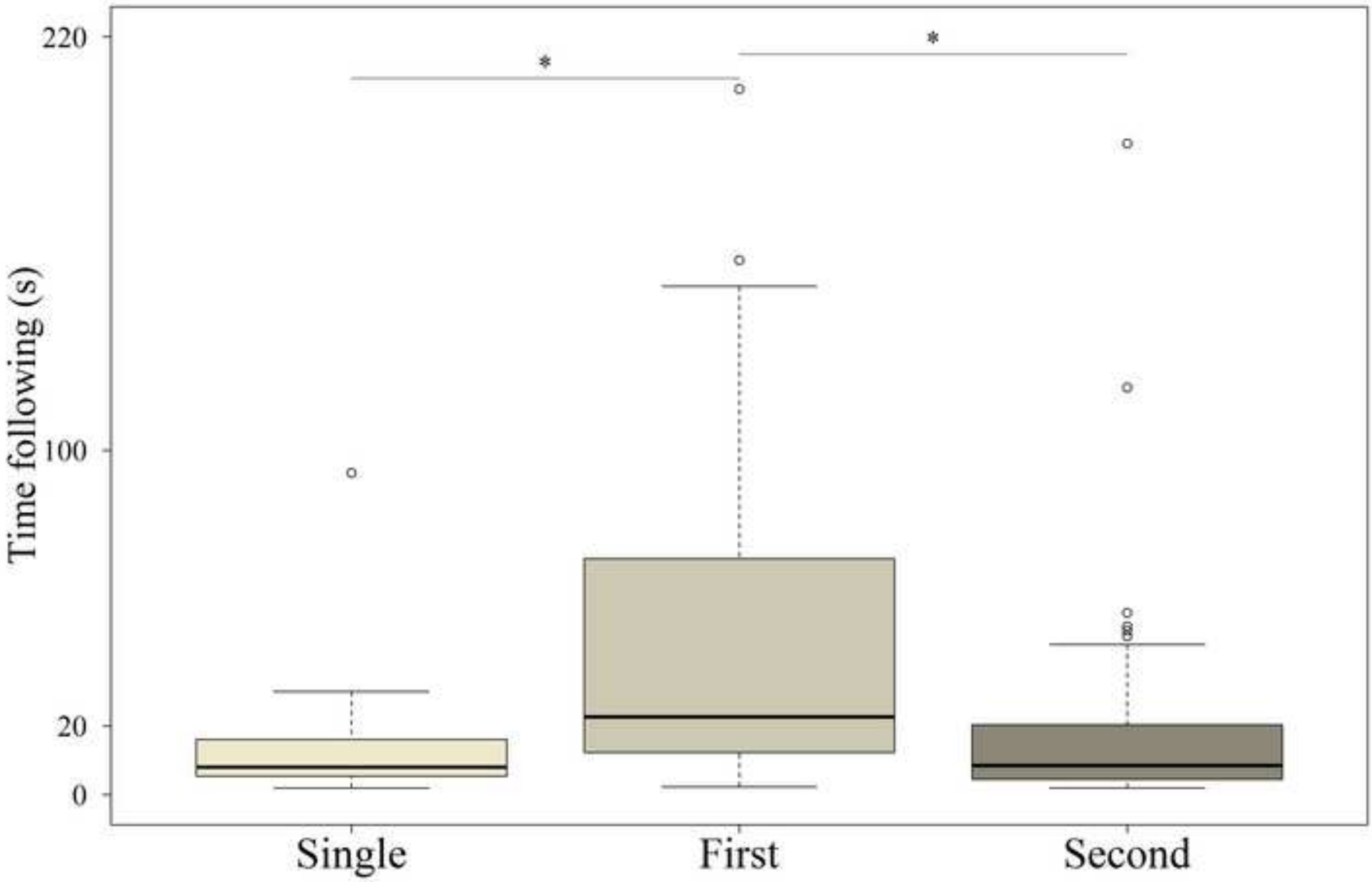


	Hypotheses			
Questions				
Males adjust to short-term order of arrival?	Yes			No
Which selective process has higher contribution to male reproductive success?	Female mate choice (precopulatory)	Sperm precedence (postcopulatory)	Both	-
	Predictions			
Male mating decisions				
(1) Approach decision (at this point only scenarios Single and Second occur)	Single	Second	Single	Single, Second
(2) Behavioural adjustment (a) Time spent following (b) Mating tactics (c) Mating behaviours sequence	Courtship display (transfer more sperm)	Unsolicited attempt (transfer sperm in last)	First	Single, First, Second

Figure



Figure

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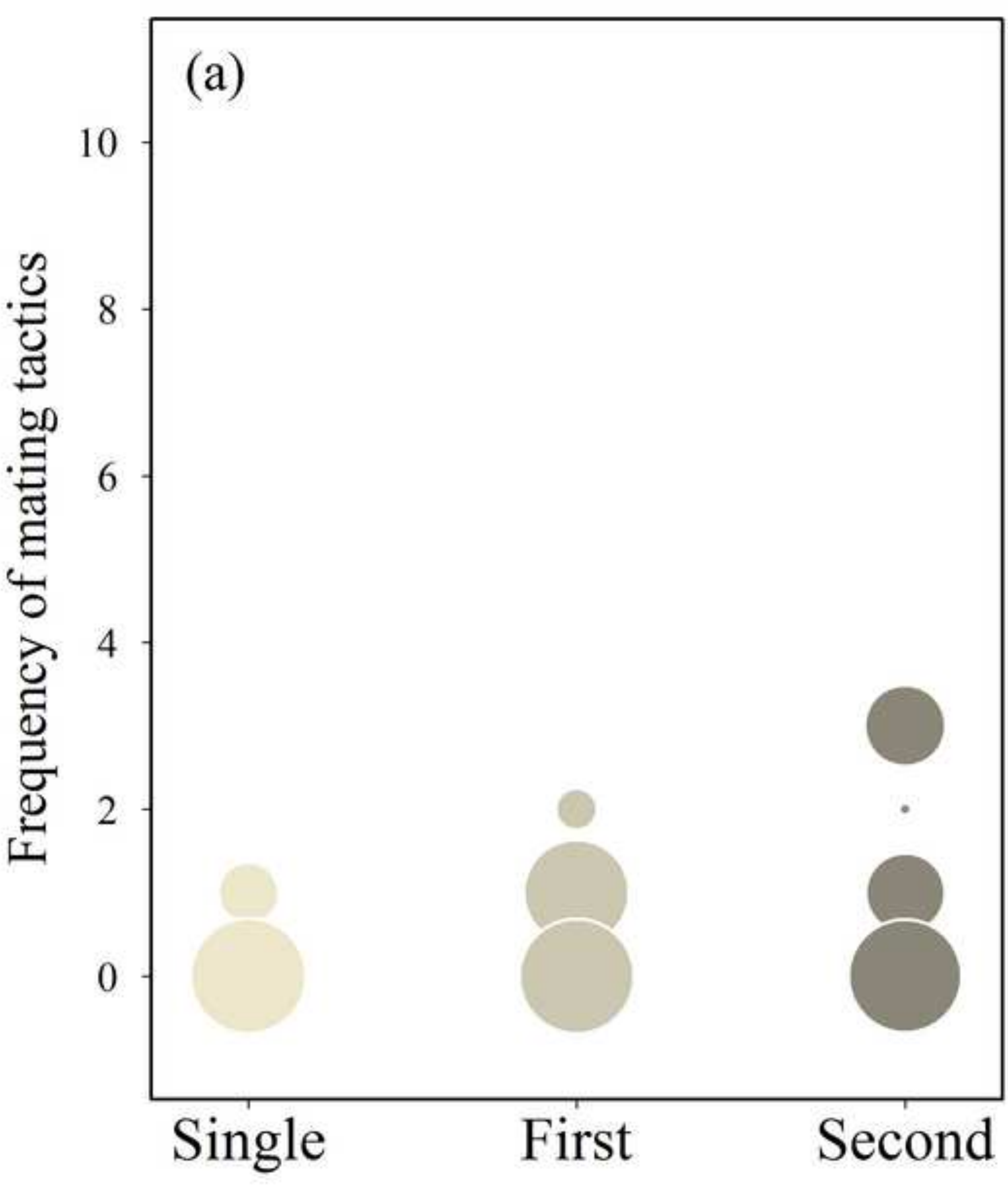

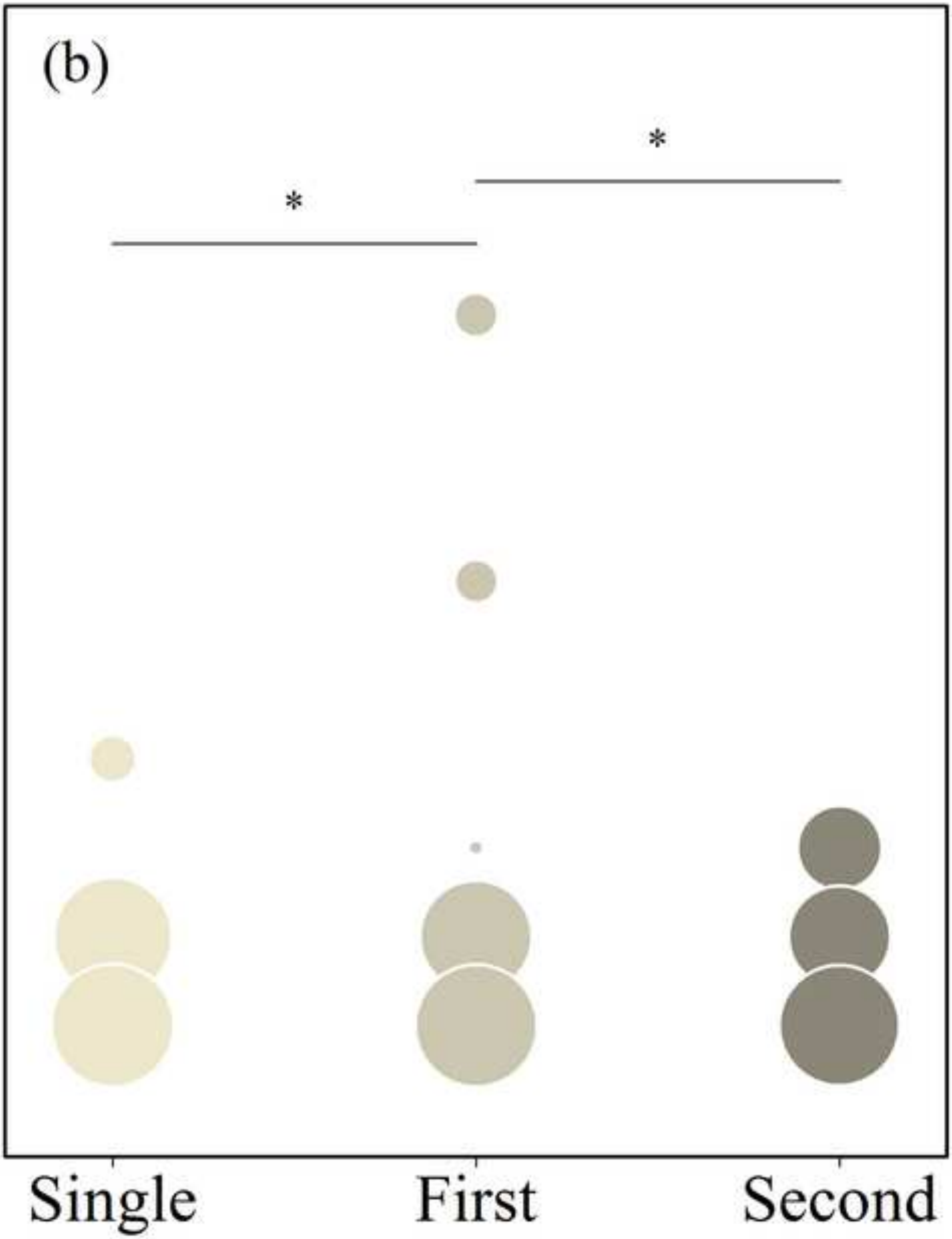
(b)
*
*
Single
First
Second

Figure

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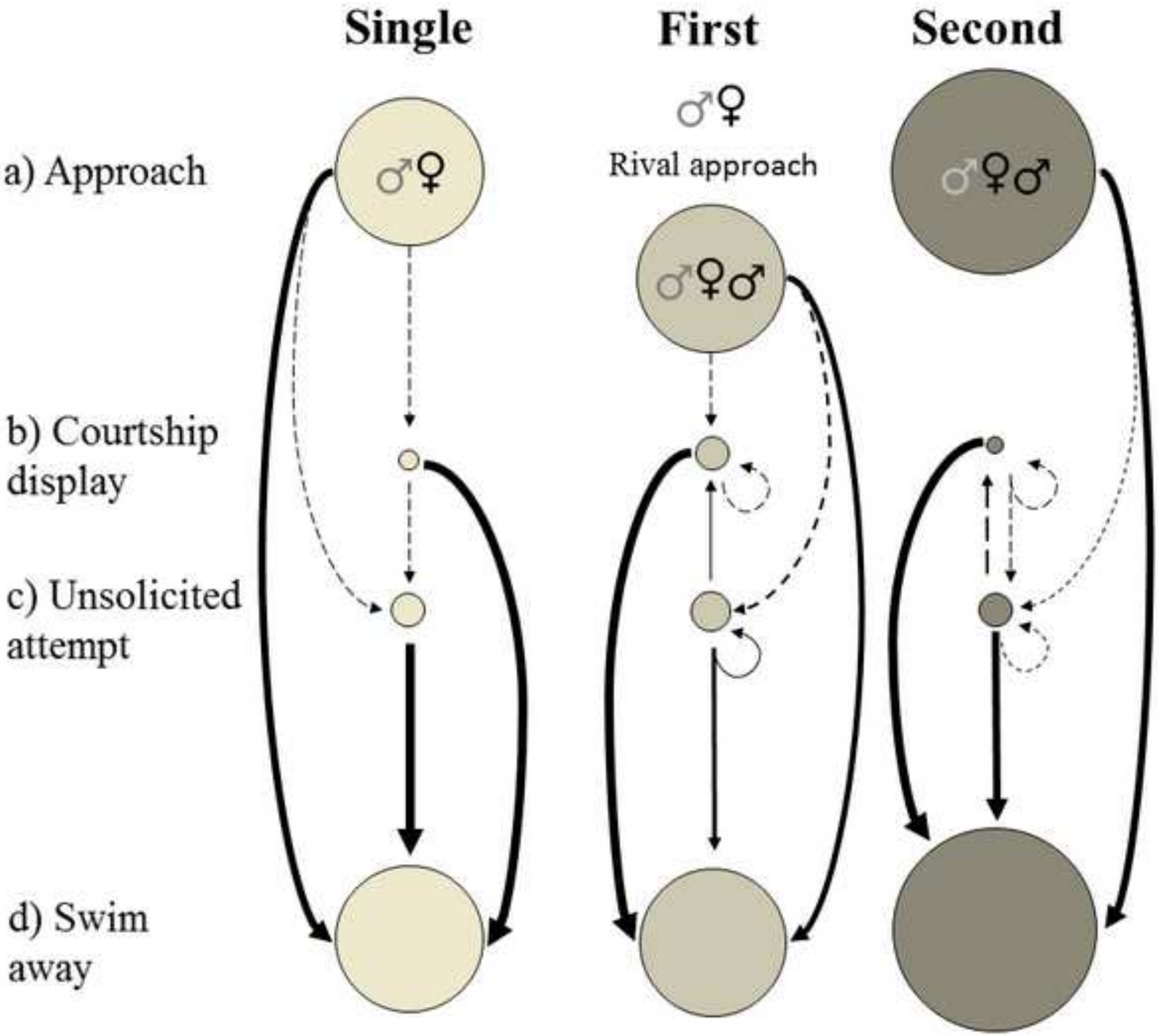
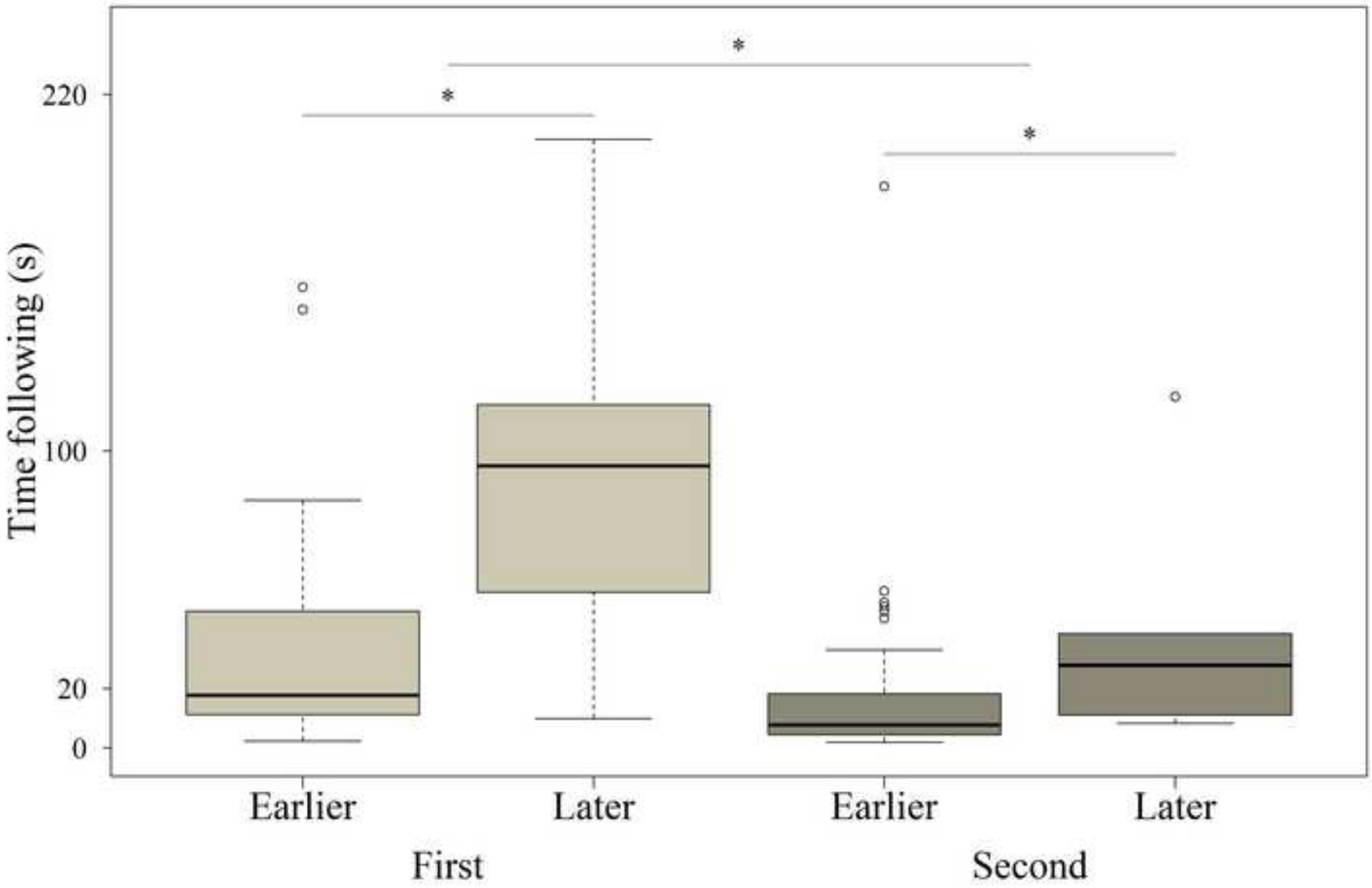
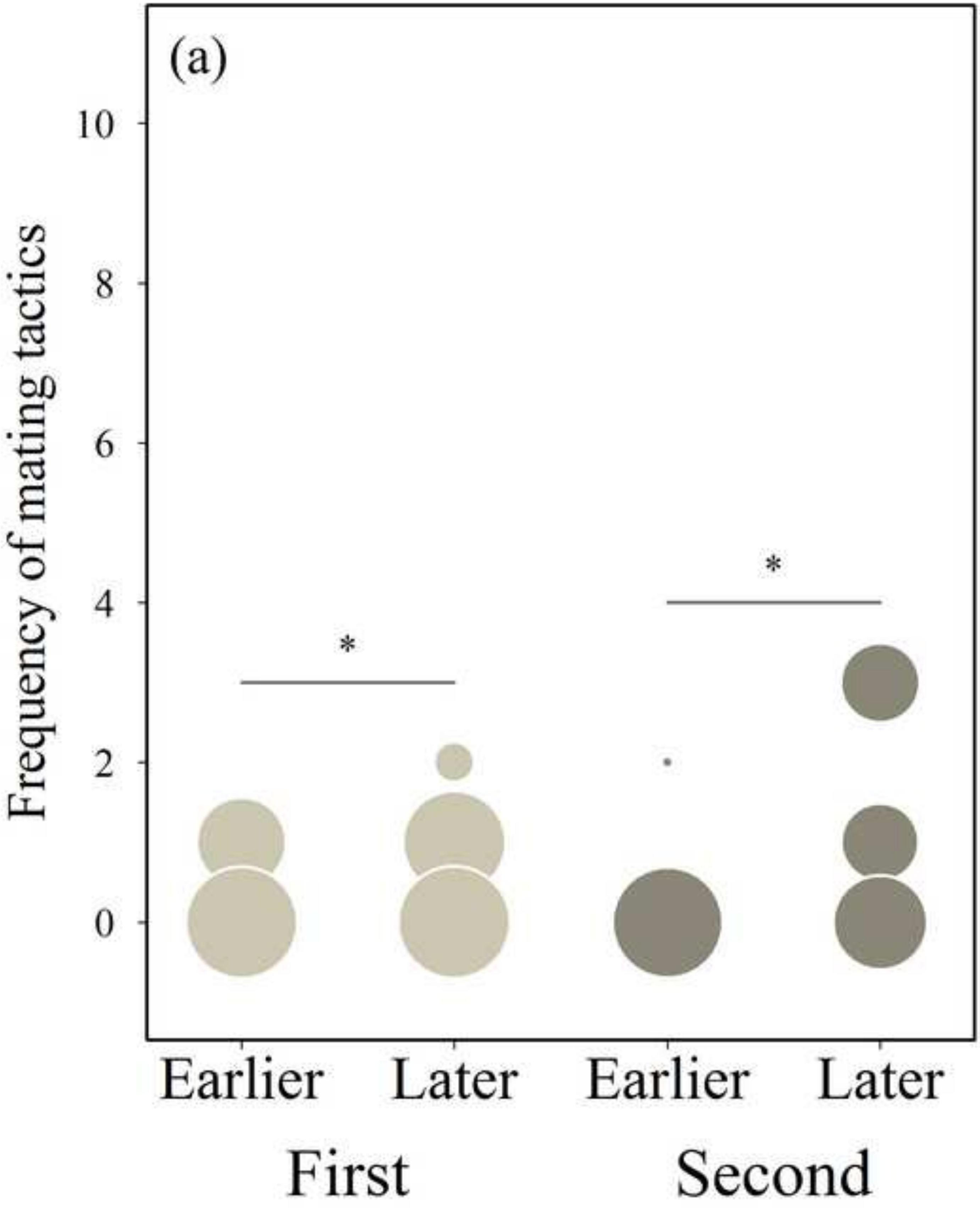
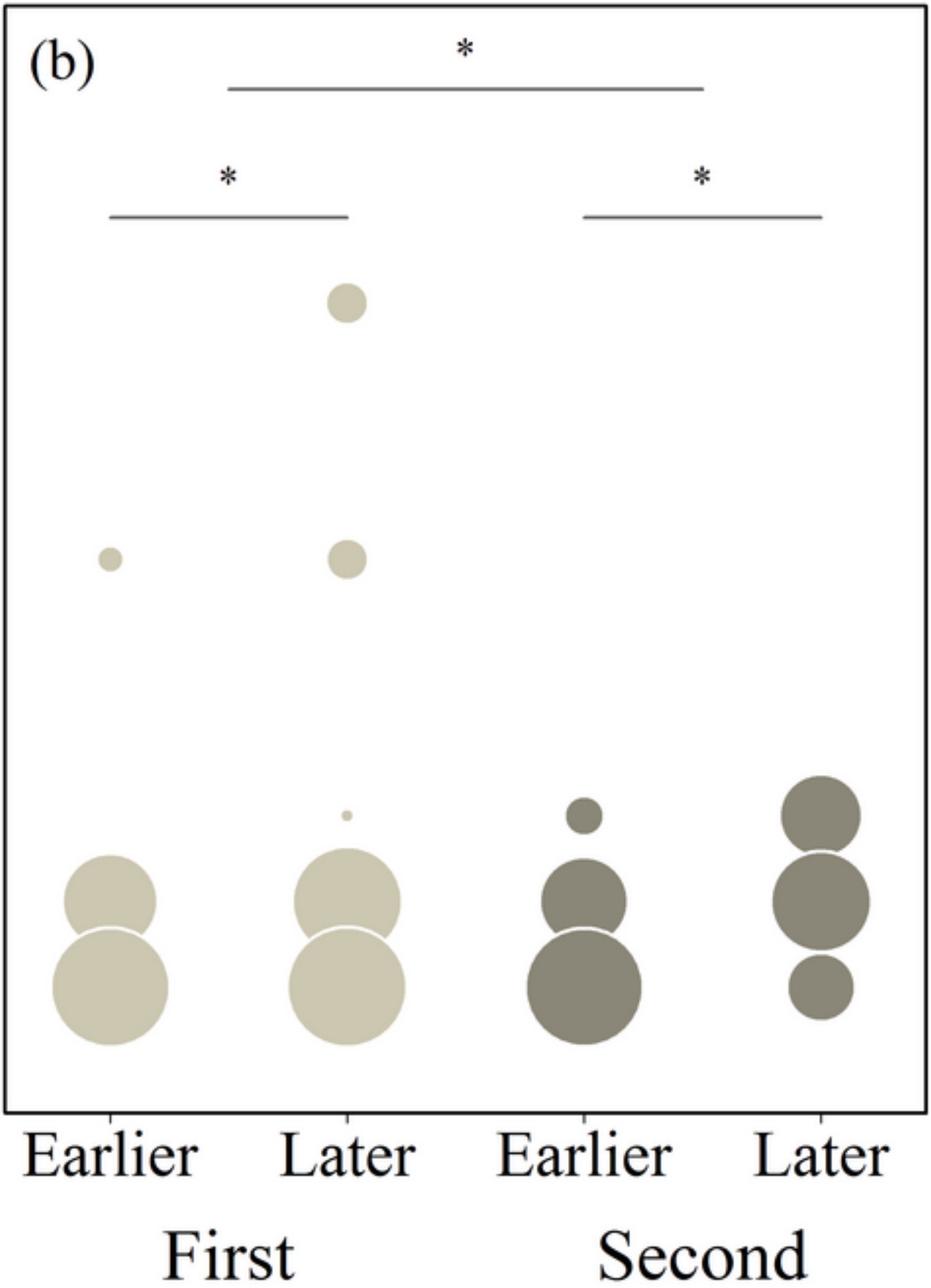



Time following (s)
220
100
20
0
*
*
*
Earlier
Later
Earlier
Later
First
Second


(a)
Frequency of mating tactics
10
8
6
4
2
0
*
*
Earlier
Later
Earlier
Later
First
Second


(b)
*
*
*
Earlier
Later
Earlier
Later
First
Second

***Animal welfare note**

Ethical notes

Before observations all individuals were kept in mixed-sex stock tanks in, with similar densities. The stock tanks had gravel in the bottom, and an aerating system. The room was kept at a controlled stable temperature. The period of light was controlled (12:12h light:dark cycle). Individuals were fed daily. Before the observations, all individuals were transferred to observation tanks (mesocosms) to acclimate to the new conditions. Female guppies are often more sensitive to changes than males, because of their stronger and more stable social bonds (Croft et al., 2006; Griffiths & Magurran, 1998). Therefore, females were transferred to the mesocosms several hours before the observations and were kept with other familiar females, while the males were transferred 15 minutes before observations started. After observations all individuals were transferred to stock tanks with no fish (labelled as “observed individuals” during the experiment). All animals were individually and carefully caught and moved between tanks with a net.

All behavioural observations were carried out at the School of Biology at the University of St Andrews. The premises where the observations were carried out comply with the UK guidelines for the treatment of animals in behavioural research and teaching, set by the UK Home Office (PCD 60/2609). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.