The effects of body size and previous experience on sexual

selection in Japanese Medaka (*Oryzias latipes*)

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Abstract

Sexual selection acts by favouring traits that give some individuals an advantage over competitors in acquiring mates and producing offspring, and more commonly acts on males due to their generally higher variability in reproductive success compared to females. There are two main mechanisms of sexual selection: competition for mates and female choice. In Japanese Medaka (*Oryzias latipes*), males can mate with many females each day, but females mate only once daily. Given that sperm counts may decrease with successive ejaculates, females should select males who have not recently mated, because higher sperm counts are positively correlated with fertilization success. Previous research has indicated that large males mate more frequently than small males because they perform better during aggressive contests, are favoured by females in choice tests, and have higher fertility following successive mating events. In addition, females may also be more receptive to males who have recently mated because mate-copying removes the need to assess a particular male. I examined the interactive effect of size and previous experience on mating behaviour and success. Each trial included a large male versus a small male, with an equal number of trials having either a large experienced male or a small experienced male. Females preferred large males in 15 of 20 trials, and, in total, 13 of 20 males that successfully mated had previous experience. Males with higher courtship and aggression rates were also more successful than males who displayed lower rates of these behaviours. Overall, males with previous experience tended to have better performance in terms of courtship intensity and mating success. This suggests that in mating systems where males have more opportunities to fertilize multiple females successively, there may be a benefit to prior mating experience.

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Table of Contents

Introduction

Sexual selection has classically been defined as "the advantages that certain individuals have over others of the same sex and species in exclusive relation to reproduction" (Darwin 1871). It differs from natural selection in that it is directly related to success during competition for mates, rather than a struggle for survival alone (Darwin 1859). Sexual selection acts by favouring traits that give some individuals an advantage over competitors in acquiring mates and successfully producing offspring (e.g., Hosken & House 2011). This type of selection acts more commonly and strongly on males than females due to their generally higher variability in reproductive success (Bateman 1948; Hosken & House 2011). Sexual selection acts via two main mechanisms: competition for mates and mate choice (Andersson 1994; Darwin 1871).

In many species, males possess certain traits which can increase their success when directly competing with another male for access to females (Andersson 1994). These traits can include exaggerated secondary sexual characteristics that are specialized for success during combat, such as large horns in many ungulates (e.g. Geist 1966; Preston et al. 2003; Valentine & Balaban 1994). In these species, horn size is a good predictor of mating success, as males with larger horns tend to obtain more mates (Preston et al. 2003). In addition to structures that are used in combat, large male body size (e.g., Bisazza & Marconato 1988; Dickerson et al. 2002; Haley et al. 1994) and/or social ranking (Le Boeuf 1974) is often associated with an advantage during aggressive contests.

Female choice can also play a key role in sexual selection, as females choose males with certain traits that confer either direct or indirect advantages (Andersson 1994).

Characteristics such as male ornaments in many species of birds are good predictors of female choice (e.g., Hill 1990), and females tend to choose males with more exaggerated colouring as this can be an indicator of male health (Hill 1990). Male traits that are favoured in competitive contests, such as large body size, may also be favoured by females (Bateman et al. 2001; Howard et al. 1998). In many species, females are the choosy sex because they invest more energy in the production of fewer, large gametes, as opposed to males who produce large quantities of smaller gametes at a lower energetic cost (Andersson 1994).

Much of the theory of sexual selection was founded in the premise that males have a limitless supply of sperm, as males of many species can fertilize the eggs of multiple females successively (e.g. Bissoondath & Wiklund 1996; Pitnick & Markow 1994; Sato et al. 2005; Weir & Grant 2010), and because sperm are smaller and less costly to produce than eggs (Bateman 1948; Dawkins 1976). In males reproductive success is positively correlated with the number of mating events, and thus they should mate as often as possible (Arnqvist & Rowe 2005). Over the past 30 years, a considerable amount of research has indicated that there are limitations to sperm supplies in several different species (e.g., Halliday 1976; Jones 1973). For example, sperm counts can decrease with successive ejaculates, and at some point following successive matings, males who were once favoured by females are no longer selected as mates (Dewsbury 1982; Nakatsuru & Kramer 1982). In theory, females should then select males who have not recently mated, because higher sperm counts are positively correlated with fertilization success (Cohen, 1971, 1975; Miller et al., 2002). For females to assess the fertility of males, they require either an honest signal of fertility (Weir & Grant 2010), or an indication of mate quality through observing a male mate with other females (Grant & Green 1996).

Japanese Medaka (*Oryzias latipes*) are an externally fertilizing species of killifish. During mating events, the male grasps the female with his large anal and dorsal fins, and they quiver together until gametes of both individuals are released (Ono & Uematsu 1957). The eggs remain attached to the female's ventral side by sticky filaments, and can be removed and collected to quantify fertilization success and for genetic assessment of paternity. Females mate once per day, but males can fertilize the eggs of up to 25 females per day, making them an ideal study species for investigating the effects of sperm depletion on sexual selection (Leaf et al. 2011).

Previous research has indicated that female medaka prefer larger males over smaller males (Howard et al. 1998; Howard et al. 2004), perhaps because they have higher fertility after increased mating events over smaller males (Howard et al. 1998). Females may also be more receptive to males who have recently mated (Grant & Green 1996) This could be because a female watches another female choose to mate with a male and can avoid the cost of assessing that male (Losey et al. 1986; Pruett-Jones 1992). Female medaka also prefer males with higher courtship rates (Grant & Green 1996), but male courtship rates decrease with increased mating events (Weir & Grant 2010). Thus, females can detect males that are sperm depleted and decrease the number of eggs they release as male courtship rates slows, suggesting that the rate of courtship behaviour provides an honest signal of fertility (Weir & Grant 2010). Female medaka can indicate a refusal to mate using a characteristic 'head-up' behaviour, indicating rejection (Ono & Uematsu 1957).

The primary objective of this research is to determine how previous experience influences sexual selection. I predict that if prior mating has a positive effect on mating success (Grant & Green 1996; Losey et al. 1986; Pruett-Jones 1992), then males with

previous experience will outcompete males without this experience during direct contests for females. Alternatively, if prior mating has a negative effect on mating success due to sperm depletion and reduced courtship (Dewsbury 1982; Nakatsuru & Kramer 1982; Weir & Grant 2010), then males with previous experience will have reduced mating success. I also predict that males who court with a higher intensity (Grant & Green 1996) and males who are more aggressive (Howard et al. 1998) will achieve greater mating success. A secondary objective of my research is to verify whether mating behaviour and success differs between small and large males. Based on previous studies, large males should have greater mating success than small males (Howard et al. 1998; Howard et al., 2004).

Methods

2.1 Experimental Animals: Marking & Housing conditions

Thirty-five juvenile and eighty mature Japanese Medaka (*Oryzias latipes*) were obtained from Aquatic Research Organisms in New Hampshire USA. All fish were anaesthetized with 0.15g/L MS222 (Tricaine S) buffered with 0.3g/L sodium bicarbonate, and tagged for individual identification using Visual Implant Elastomer tags (NorthWest Marine Technology) at least one week prior to the start of experiments. Each fish was tagged in two locations out of a possible four (on the back, either anterior or posterior to the dorsal fin, on either the right or left side), using one or two of four possible colours (pink, green, black or blue). The same tagging procedure was used on juvenile fish after sexual maturation. Fish were weighed to the nearest 0.001g and standard length measured to the nearest 0.01mm using digital calipers. Caudal fin clips were cut from each fish using scissors, and placed in 90% ethanol for later DNA analysis. All fish were placed into six stock tanks (two 20 gallon tanks measuring 61cm x 30cm x 40cm and four 15 gallon tanks measuring 50cm x 25cm x 45cm) at a sex ratio of roughly three males: five females. The tanks contained fresh water (salinity 0.25ppt), an undergravel filter, were kept at a temperature of 26°C-28°C, and a photoperiod of 14hr light:10hr dark with lights on at 0815h. Mating occurs naturally in the stock tanks, and fish were fed twice daily using a combination of commercial flakes, *Artemia nauplii*, and adult *Artemia*.

2.2 Experimental Design

In this experiment, I tested the effects of previous experience on male mating success between large and small males. I had two experimental treatments: 1) large experienced male versus small male with no previous experience, and 2) small experienced male versus large male with no previous experience. I selected males for each trial based their length, with an average length difference between males of 4.60mm, and a minimum length difference of 1.53mm. The average length of small males used was 25.20 ± 3.23mm, and the average length of large males used was 29.80 ± 2.06mm. The largest females from the population were used as focal females (0.327g - 0.658g, 27.39mm - 33.43mm in length).

To ensure that the fish in each trial did not have any previous interactions with one another, fish in each of the four 'roles' (i.e., experienced male, 10 females with whom the experienced male gained experience, focal female and other male with no previous experience) were always obtained from different stock tanks. Males and focal females were re-used in different trials, but never in the same combinations to ensure that individuals were not familiar with one another. Individuals were taken from stock tanks and placed in their respective compartments in the experiment tanks the day before the trial began so that they had no previous mating experience on the day of the trial.

2.3 Experiments

2.3.1 Experiment Tanks

Experiment tanks were divided into four compartments using screens, and kept at the same temperature and light conditions as the stock tanks. The first compartment held

the male who could mate prior to focal observations (i.e., have previous experience) , the second held 10 females, the third held a focal female, and the fourth held the other male (Figure 1). Water could move between compartments, but I rarely witnessed interactions among fish across the screen partitions.
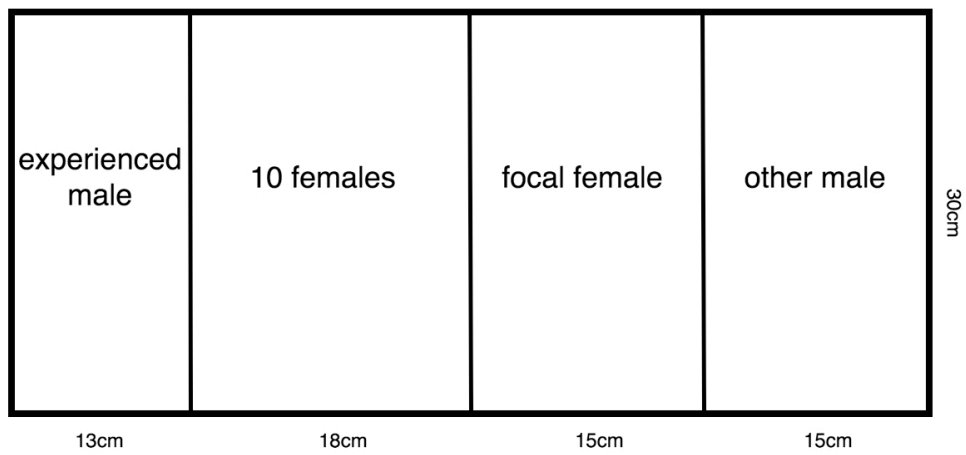
experienced male	10 females	focal female	other male	
13cm	18cm	15cm	15cm	30cm

Figure 1. Experimental tank design.

2.3.2 Experimental Procedure

The day before a trial, thirteen fish were placed into a 15-gallon (61cm x 30cm x 30 cm) experiment tank. Fish were fed 20 minutes prior to the start of each trial. During the first part of a trial, one male had access to ten females for two hours (the *Experience Phase* described below). Immediately after the two hour time period, he was placed in compartment for 30 minutes with one female (the 'focal' female) and one other male who had not yet mated (the *Competition Phase* described below). In both treatments, the experienced male would be paired against a male of the opposite size, so that there would always be a large male versus a small male in the focal female mate choice portion of the experiment.

2.3.3 Experience Phase

The experience phase of the trial was initiated by removing the screen between one of the males and the 10 females. This male was given access to the 10 females for two hours. During this period, the frequency of courtship behaviour by the male was recorded. Courtship was expressed as quick circles, which occur when the male swims in a fast arc in front of the female (Grant et al. 1995). After each mating event, I recorded the time and removed the female from the tank using a plastic container. Females who mated were held in a 1L container until egg collection. Eggs were taken from the female's ventral side with a small paint brush, and placed in a petri dish with rearing solution (1.0g NaCl, 0.03g KCl, 0.04g $CaCl_2 \bullet H_2O$, 0.163g $MgSO_4 \bullet 7H_2O$, 10ml 0.01% Methylene Blue, distilled water to 1L). After the trial, each female's eggs were examined under a dissecting microscope for fertilization. Egg fertilization was determined by visual

observation using a dissecting microscope, as fertilized eggs can be identified by a perivitelline space that develops between egg membranes (Iwamatsu et al. 1991). Males had between two to seven successful mating events (with an average of four) during this part of the trial.

2.3.4 Competition Phase

Immediately following sperm depletion, the screen between the focal female and the male who had not yet mated was removed, and the male with previous experience was added to the compartment simultaneously. Interactions among these three fish were observed for 30 minutes or until spawning occurred. I video recorded this part of the trial using an Olympus TG-4 camera fixed to a tripod. During each trial, male-male aggression and male courtship rates were determined for both males because the rate of these behaviours can affect their success, and courtship rate can change with an increase in mating events (Weir & Grant 2010). Aggression was defined as the occurrence of a chase, which occurred when one fish quickly charges at another, causing the other to either flee or be hit by the charging fish (Grant et al. 1995); courtship was defined as above for the *Experience phase*. Refusal to mate by the focal female was assessed through 'head up' displays, which occur when the female tilts her head vertically upward in the water column (Hamilton et al. 1969). Aggression, courtship and head-up displays were quantified from video recordings.

2.4 Statistical Analysis

Prior to analysis, counts of courtship, aggression and refusal were standardized into rates per minute because the duration of competition observations varied depending

upon how quickly mating occurred. I used generalized linear models to test the interactive

effects of male size and previous experience on male mating behaviour. Similarly,

generalized linear models were used to compare mating behaviour between successful

and unsuccessful males, as well as the interaction between success and body size. Lastly,

generalized linear models were used to test the relationship between female refusal rate

and the interaction between male size and mating success (i.e., successful mating with a

female), as well and between female refusal rate and the interaction between male size

and previous experience. I used binomial tests to compare whether the probability of

success differed between large and small males, as well as males with and without

previous experience. All analyses were done using R Studio V1.0.136 (R Core Team,

2016).

Results

The focal female mated in 20 of 36 trials. The following results focus on these 20 trials, 10 of which had large experienced males and 10 had small experienced males.

3.1 Experience Phase

Males mated with 1-7 females (mean ± standard error (SE)= 4.3 ± 0.6) in the experience phase, with clutch sizes ranging from 1-22 eggs (mean ± SE= 8.43 ± 0.53). Large males mated with 2-7 females (mean ± SE = 4.8 ± 1.5), with clutch sizes ranging from 1-23 eggs (mean ± SE = 9.9 ± 5.3). Small males mated with 2-6 females (mean = 3.8 ± 0.5), with clutch sizes ranging from 1-14 (mean = 6.5 ± 3.9). The average large male courtship rate during the experience phase was 1.56 ± 0.17 quick circle displays per minute and the average small male courtship rate was 1.67 ± 0.11 displays per minute. On average, large males fertilized 68.24 ± 4.67% of a clutch of eggs in the experience phase, and small males fertilized 71.67 ± 5.20%. Data from the experience phase for each trial is summarized in Appendix Table 1.

3.2 Competition Phase

3.2.1 Mating and Fertilization Success

Of the 20 trials with successful matings during the competition phase, large males were significantly more successful than small males, with 15 large successful males, and 5 small successful males (binomial test, p = 0.04). Of the 20 trials with successful

matings, 13 were by experienced males (binomial test, p = 0.26). Nine of the 13 males were large males and 4 were small males.

Female clutch sizes ranged from 3-26 eggs (mean = 11.45 ± 0.56). Males with previous experience fertilized clutch sizes ranging from 3-19, and males with no previous experience fertilized clutch sizes ranging from 10-26. Large experienced males had an average fertilization success of 75.9 ± 9.1% and small experienced males had an average fertilization success of 78.9 ± 5.3%. Fertilization success was 74.8 ± 12.0% for large males with no previous experience and 90% in the small males with no previous experience. Because only one small male with previous experience mated in the competition trials, we could not do a statistical comparison of these data.

3.2.2 Male Behaviour

There was no interaction between male size and success at matings in relation to courtship (Generalized Linear Model (GLM): $F_{1,36}$ = 1.42, p = 0.24). However, males who successfully mated in the competition phase had higher courtship rates than males who were unsuccessful (GLM: $F_{1,37}$ = 21.57, p < 0.001; Figure 2). This pattern was similar for both large and small males (GLM: $F_{1,38}$ = 2.11, p = 0.15; Figure 2). Males with previous experience tended to court more frequently than males without previous experience (GLM: $F_{1,37}$ = 3.85, p = 0.057; Figure 3); this was true for large and small males (GLM: $F_{1,37}$ = 1.46, p = 0.233; Figure 3). Successful males did not display higher aggression rates during the competition phase (GLM: $F_{1,37}$ = 2.71, p = 0.11; Figure 4), but large successful males had higher aggression rates than small successful males (GLM: $F_{1,38}$ = 14.09, p = 0.001; Figure 4). There was no difference in aggression rates between

experienced and inexperienced males (GLM: $F_{1,37} = 0.58$, $p = 0.45$; Figure 5). Overall,

large males had higher aggression rates than small males (GLM: $F_{1,38} = 12.51$, $p = 0.001$;
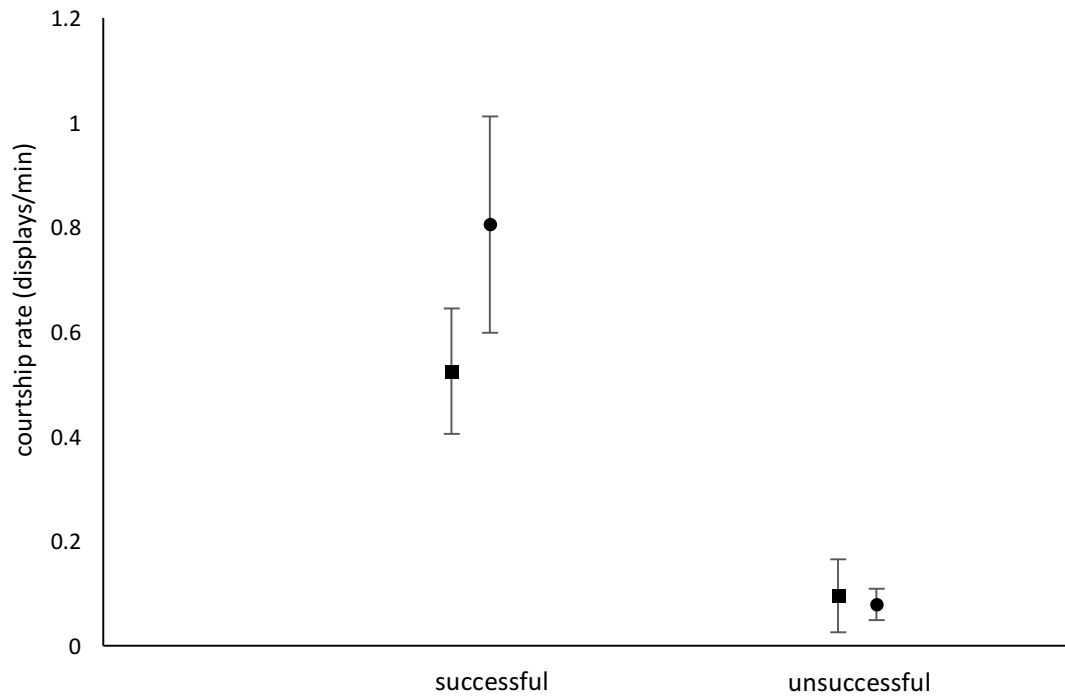
Figure 5).

Figure 2. Courtship rates (quick circle displays/minute) of successful and unsuccessful males in the competition phase. Large males are represented by squares, and small males are represented by circles. Error bars depict standard errors.
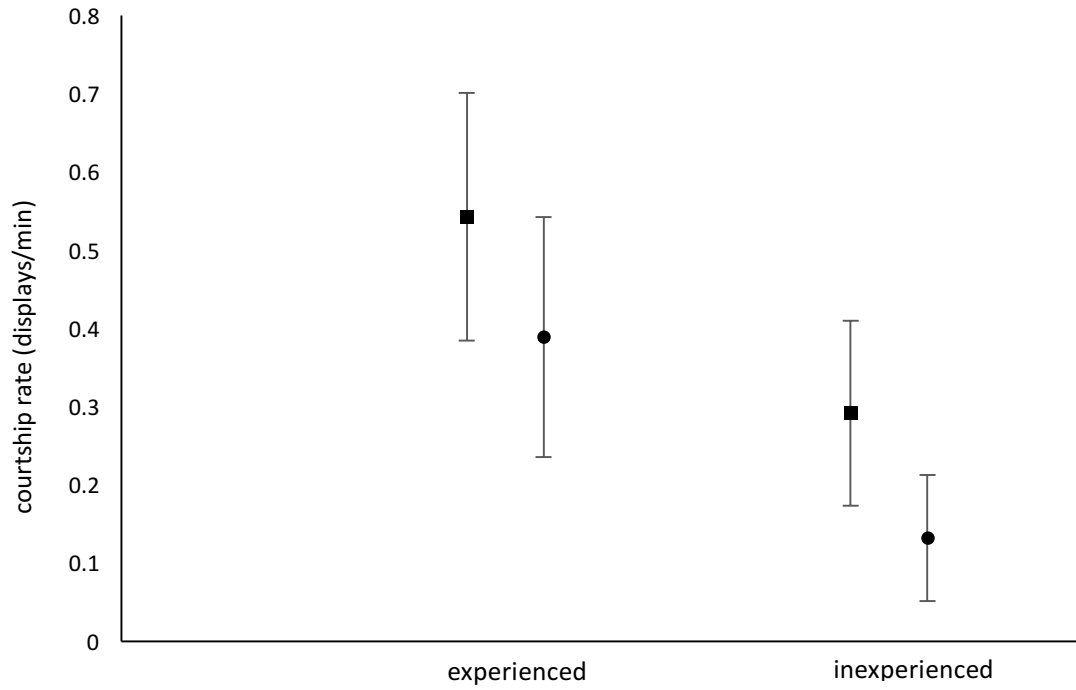
Figure 3. Courtship rates (quick circle displays/minute) of males with and without previous mating experience. Large males are represented by squares, and small males are represented by circles. Error bars depict standard errors.

Figure 4. Aggression rates (chase displays/ minute) of males who were successful in the competition phase, and who were unsuccessful. Large males are represented by squares, and small males are represented by circles. Error bars depict standard errors.
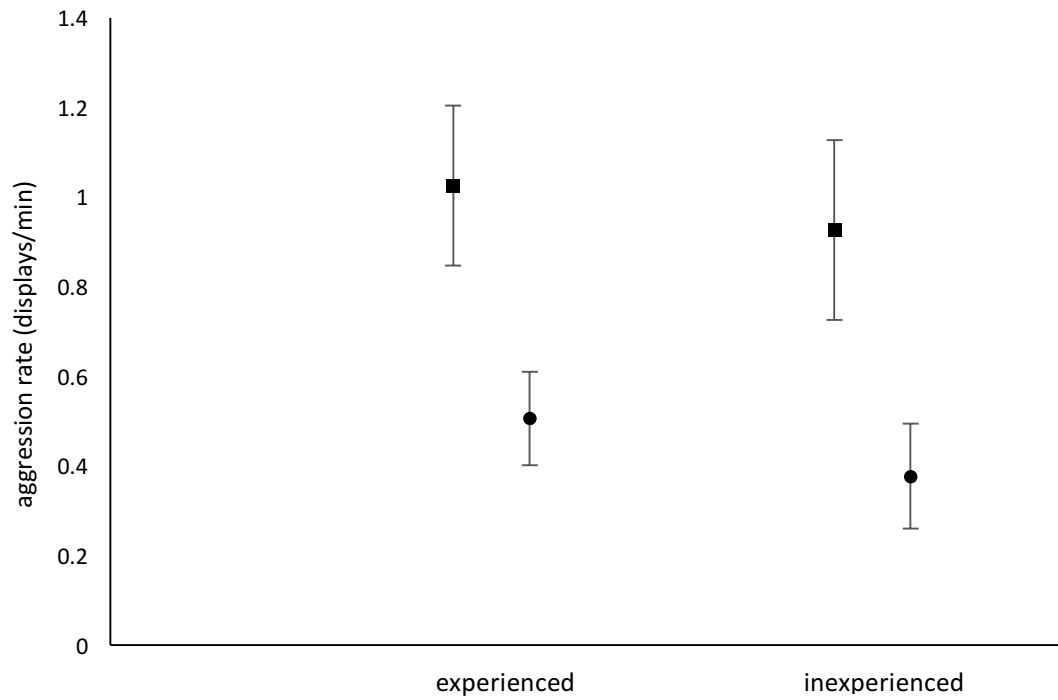
Figure 5. Aggression rates (chase displays/minute) in males with and without previous mating experience. Large males are represented by squares, and small males are represented by circles. Error bars depict standard errors.
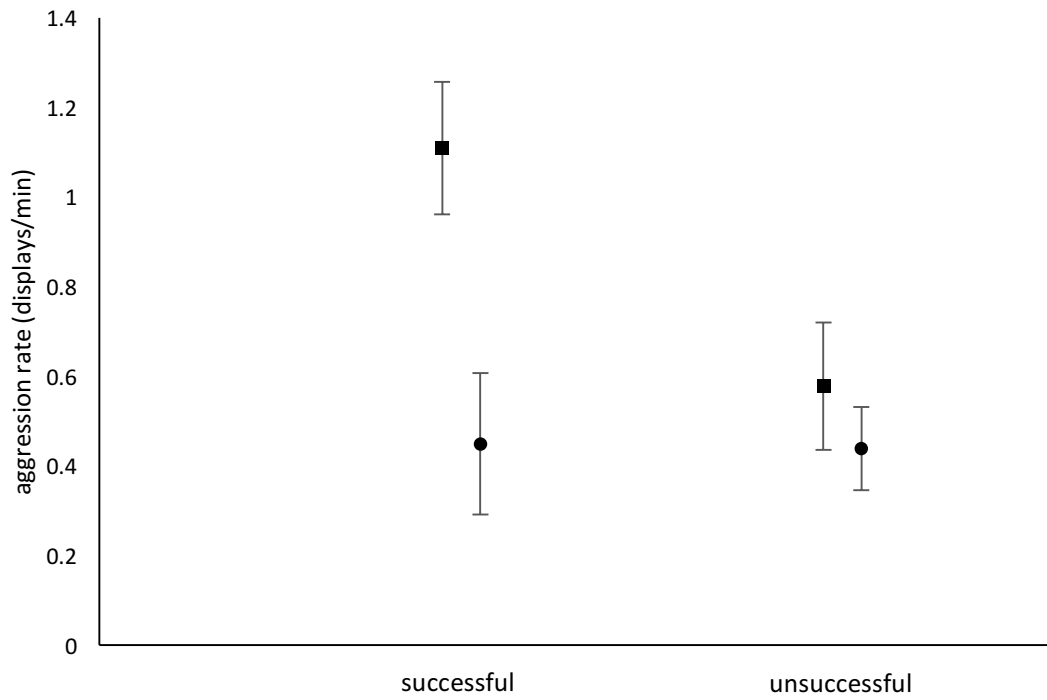
3.2.3 Female Behaviour

Focal females tended to have higher refusal rates in trials with successful small males compared to trials with successful large males (GLM: $F_{1,18} = 3.10$, $p = 0.095$), but did not show a marked trend in refusal rates when comparing successful males with and without previous experience (GLM: $F_{1,17} = 1.35$, $p = 0.26$).

Discussion

Successful males within a size class were those that courted most frequently, suggesting that this behaviour is an important determinant of mating success. My results may also suggest that male *Oryzias latipes* with previous experience are more successful in acquiring females. This was true for both large and small males, although the sample size for small males was limited as large males were successful in most of the trials.

Large males acquired more females partly due to the fact that they were more aggressive than their smaller competitors. Male body size may not be a heritable trait in Japanese Medaka (Howard et al. 1998), so males rely on their ability to compete using aggressive behaviour to win access to females (Hamilton et al. 1969; Walter & Hamilton 1970), and larger males generally tend to be better competitors over smaller males (Bisazza et al. 1989; Hoelzer 1990). Large males also tend to maintain a higher fertilization rate after successive mating events than small males (Howard et al. 1998).

Males who courted more were more successful in mating, consistent with previous research (e.g., Farr 1980; Grant et al. 1995; Grant & Green 1996; Reynolds 1993). Focal females did not observe experienced males mating with other females in the experience portion of the experiment, so these results could not be associated with mate copying concepts (as in Howard et al. 1998). Further investigation into females' ability to detect previous experience without physical observation is needed to explain this.

Courtship is an honest signal of fertility (Weir & Grant 2010), which may explain why females are more receptive to males who display higher courtship rates. Despite evidence that males with previous experience may reduce mating behaviours (van Son & Thiel 2006; Weir & Grant 2010), males with previous experience tended to court more in

my study (as in Hoefler et al. 2010). Courtship latency, defined as the time elapsed from introduction to the first courtship display (Eastwood & Burnep 1977), could be one explanation for male success, as males with reduced courtship latency may have higher mating success (Hoefler et al. 2010). Inexperienced males who initiate courtship sooner may be more successful, but males with previous experience (and especially large males) may have reduced courtship latencies (Dukas 2005; Hoefler et al. 2010). Another explanation for higher courtship rates in experienced males could be that 'winning' behaviour in the experience phase of the experiment could enhance sexual motivation in these males (Dyakonova & Krushinsky 2008).

Small and large male sizes, as well as the fertilization results from the experience phase of this experiment were similar to Howard et al. (1998) and did not show a marked decrease in fertilization success that was observed in other studies (Weir and Grant 2010). While I expected males to mate with all ten females in this part of the experiment, the maximum number of matings during this phase was 7. Thus, these males were not necessarily sperm depleted, but were nonetheless in a situation where they invested energy in courtship behaviour and egg fertilization before the competition phase began. My results for the experience phase of the experiment suggest that there is no difference in fertilization success between large and small males, a finding consistent with that of Weir and Grant (2010).

Of more general interest is the fact that males with previous experience tended to exhibit better performance in the form of greater courtship intensity and mating success compared to males without previous experience. This suggests that previous experience plays an important role in determining subsequent mating behaviour, which results in increased mating success. Thus, in mating systems where males have the opportunity to

fertilize the eggs of multiple females per day, there may be benefits of increased mating

events not only in the form of increasing the total number of offspring, but also an

increased probability of success due to enhanced mating behaviour, especially for smaller

males.

Acknowledgements

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Appendix

Table 1. Clutch size and egg fertilization data of each successive mating event from the *Experience Phase* of the experiment, including male and female standard length (mm) and courtship rate (quick circle displays/ minute). * Two clutches from trial ten could not be examined.

trial	male standard length (mm)	courtship rate (quick circle displays/minute)	female standard length (mm)	clutch size	% fertilization
1	30.86	1.41	27.69	13	15.38
		1.13	24.81	8	12.5
		0.87	29.12	14	0
		2.11	28.85	4	0
2	25.13	2.00	27.27	7	100
		1.94	28.81	14	100
		1.29	28.95	7	85.71
		2.00	27.44	12	58.33
		1.50	27.82	10	70
		1.43	29.59	8	100
3	29.37	2.00	26.34	14	100
		1.38	29.39	11	100
		1.91	28.82	5	100
		2.62	28.49	6	83.33
4	21.12	0.29	26.92	2	50
		1.16	26.49	6	0
5	25.28	2.03	24.81	6	100
		2.16	29.12	9	100
		2.13	28.75	2	50
		2.23	27.47	3	100
6	27.57	2.07	28.82	1	100
		2.67	28.49	10	90

trial	male standard length (mm)	courtship rate (quick circle displays/minute)	female standard length (mm)	clutch size	% fertilization
7	31.64	0.91	33.43	21	95.24
		1.67	26.92	5	100
		1.45	28.82	11	100
		1.59	25.54	6	100
		1.67	28.68	11	100
		1.29	27.96	3	66.67
8	26.98	0.33	29.39	6	66.67
		1.10	26.34	6	100
9	26.25	1.70	27.44	5	100
		2.29	27.82	7	100
		3.13	29.08	9	88.89
		2.20	29.81	5	100
10	24.88	0.25	28.85	0	NA*
		1.51	25.54	6	100
		2.15	26.92	5	80
		1.50	33.43	0	NA*
		1.00	28.68	12	100
		1.80	27.96	3	0
11	29.68	1.69	24.81	17	88.24
		1.24	27.69	10	90
		1.64	29.12	13	100
		2.94	28.52	15	86.67
		1.86	29.25	4	50
		2.38	26.77	3	66.67
		2.10	28.85	11	90.91
12	29.37	0.50	27.82	17	58.82
		1.20	30.32	23	95.65
		0.75	26.71	5	40
		0.67	29.59	9	77.78
		0.53	27.44	13	76.92
		0.32	29.08	8	62.5

trial	male standard length (mm)	courtship rate (quick circle displays/minute)	female standard length (mm)	clutch size	% fertilization
13	23.46	1.45	24.81	13	15.38
		2.13	29.25	8	37.5
		1.86	27.69	8	62.5
		1.53	26.77	3	33.33
		0.00	30.32	26	96.15
14	30.47	0.67	28.82	14	42.86
		0.67	29.81	5	100
		1.81	26.34	2	0
		2.36	28.99	15	86.67
		1.67	25.08	1	0
		0.00	28.85	14	100
15	31.64	1.72	25.54	7	71.43
		3.14	26.92	3	33.33
		2.21	28.85	15	100
		1.50	33.43	22	86.36
		0.00	29.12	9	100
16	25.13	1.21	28.82	7	57.14
		1.42	29.81	11	36.36
		1.00	26.34	3	66.67
		2.05	28.99	12	91.67
		0.00	33.43	16	93.75
17	30.47	1.09	29.59	9	66.67
		8.14	27.44	9	66.67
		0.00	28.99	8	37.5
18	30.47	0.58	28.49	8	87.5
		1.63	27.44	11	81.81
		1.36	30.32	10	0
		1.25	29.08	7	85.71
		2.22	27.82	6	66.67
		0.94	29.59	5	100
		0.00	28.82	3	100
19	26.25	1.00	29.12	5	20
		0.86	24.81	10	0
		2.69	27.69	5	80
		2.32	28.85	2	50
		0.00	27.82	7	71.43

trial	male standard length (mm)	courtship rate (quick circle displays/minute)	female standard length (mm)	clutch size	% fertilization
20	29	2.93	25.54	18	55.56
		1.31	27.44	11	72.73
		1.50	29.08	10	70
		1.40	27.69	9	44.44